ON THE AUTOMATION OF SOLID SURFACE ROOM TEMPERATURE PHOSPHORIMETRY AND ITS APPLICATION AS A DETECTION TECHNIQUE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

BY

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ON THE AUTOMATION OF SOLID SURFACE ROOM TEMPERATURE PHOSPHORIMETRY AND ITS APPLICATION AS A DETECTION TECHNIQUE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Solid Surface Room Temperature Phosphorimetry (SSRTP) is an analytical technique that has been growing as a promising tool for the analysis of trace components in biological, pharmaceutical and environmental samples. Although it is a simpler technique to work with than low temperature phosphorimetry, the manual sampling procedure which is normally used to perform SSRTP analysis is time consuming and therefore, inconvenient for applications in areas where numerous samples are handled routinely.

Few attempts have been made to develop an instrumental system for the automation of SSRTP. In these attempts, the manual steps were minimized to spot the solutions onto the solid substrate and optimize the position of the phosphor spot in the sample compartment of the spectrofluorimeter.

These studies involve the development of a new automatic system which employs a concentric nebulizer to spray the analyte solution onto the solid substrate. Since the manual steps were completely eliminated, the new system made SSRTP less dependent on the investigator's expertise.

Short analysis times and satisfactory analytical figures of merit (AFOM) showed the applicability of the technique for routine analysis

The versatility of the new system permitted its use in flowing systems Flow injection analysis (FIA) was employed as a continuous sample introduction technique in such a way that the tedious dilution process involved in batch procedures was substituted by a rapid and easy way of obtaining calibration curves

The automatic system was then proposed as a selective permanent record detector for high performance liquid chromatography (HPLC) The chromatographic parameters and the AFOM of five well known phosphorescent compounds were compared to those obtained by UV detection Overlapped compounds were individually identified, demonstrating that the selectivity of the SSRTF detector can be a useful feature in case of incomplete separation of complex mixtures.

Finally the proposed detector was employed in the HPLC analysis of caffeine, theophylline and theobromine The AFOM and the chromatographic parameters obtained with the SSRTF detector were compared to those obtained by UV absorption detection The SSRTF and UV chromatograms of spiked urine samples were compared and the feasibility of keeping an analyzed sample on the filter paper strip as a permanent record was discussed

CHAPTER 1
INTRODUCTION TO PHOSPHORIMETRY

Excited State Processes

Photoluminescence spectroscopy is based on the detection of radiation of 200-700 nm emitted during the deactivation of electronically excited molecules [1-3]. Under normal conditions, the orbitals of lowest energy of an organic molecule are occupied by pairs of electrons with spin in opposite directions [4]. Since most of the organic molecules have an even number of valence electrons, the resulting electron spin is zero. Such a state, with no net spin, is called the singlet state. The singlet state of lowest energy is known as the ground state, and it is represented in the Jablonski diagram of Figure 1-1 by S_0. Each electronic state has several vibrational levels which, under different conditions, can represent the energy state of a molecule. At room temperature, most of the molecules are in the lowest vibrational level of the S_0 state.

Through the absorption of electromagnetic radiation (A), a molecule can pass from the ground state to an excited state of higher energy. This transition occurs in approximately 10^{-15} s and entails the promotion of an electron from the highest occupied orbital to a previously unoccupied one. If the transition occurs with no change in the spin of the promoted electron, the excited state will have two unpaired electrons with antiparallel spins and, therefore, net spin equal to zero. An electronic state with these characteristics is known as a singlet excited state. In Figure 1-1, the first and the second singlet excited states are represented by S_1 and S_2, respectively. If the transition involves a change in the electronic spin, the excited state will be characterized by two unpaired electrons with parallel spins. In this case, the resultant spin is one, and the excited state receives the name of triplet state. In

Figure 1-1 Jablonski diagram showing the different radiative and nonradiative processes that can occur in a molecule upon excitation into a singlet state. S_n (A absorption, F fluorescence, IC internal conversion ISC intersystem crossing P phosphorescence VR vibrational relaxation) From reference 4

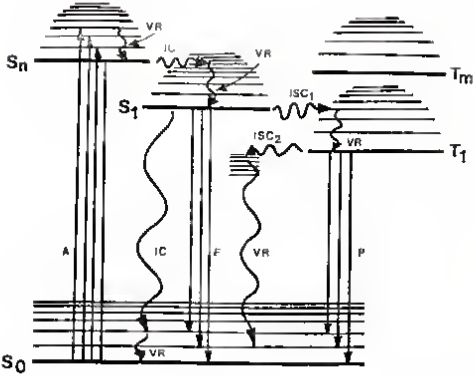

VR
IC
VR
S_n
T_m
S_1
ISC_1
ISC_2
VR
T_1
A
IC
F
VR
P
VR
S_0

Figure 1 1: the triplet state of lowest energy is symbolized by T_1, while any other triplet state of higher energy is represented by T_n.

When the energy of an absorbed photon is enough to excite a molecule to a state such as S_2, it usually releases the extra vibrational energy to reach the lowest vibrational level of the state. This radiationless deactivation mechanism is known as vibrational relaxation (VR) and it is the consequence of the energy transfer from the excited molecule to the surrounding medium in the form of thermal energy. Vibrational relaxation involves vibrational levels of the same electronic state, and it is faster than any other electronic transition (on the order of 10^{-13} s or less) [3,4].

Through another radiationless process called internal conversion (IC), the molecule passes from the lowest vibrational level of S_2 to the highest vibrational level of S_1. This process is the result of the transformation of exciting energy into vibrational-rotational energy [5] and occurs between electronic states of the same multiplicity [6]. The lowest vibrational level of S_1 is then reached through VR. If the molecule was initially excited to a higher excited state than S_2, the lowest vibrational level of S_1 would be reached by a succession of IC and VR processes. From the lowest vibrational level of S_1, the molecule has two ways of directly returning to the ground state. Through IC without the emission of radiation, or by the emission of a photon with no change in the electronic spin. The latter process is responsible for the emission of fluorescence and occurs in a period of time of 10^{-9} to 10^{-6} s. The energy of the emitted photon corresponds to the energy gap between the lowest vibrational level of S_1, and the ground state. When the lowest vibrational level of the S_1 state overlaps with S_0, the excited state is deactivated by nonradiative relaxation and the emission of fluorescence does not occur.

When the emitted photon has the same energy as the one initially absorbed, the process is termed resonance fluorescence. Most times however, the energy loss in VR and IC results in the emission of

fluorescence at longer wavelengths than the excitation wavelength, and resonance fluorescence is not observed.

The remaining possibility to return to S_0 from S_1 begins with a process called intersystem crossing (ISC). ISC is a radiationless mechanism involving systems of different multiplicity which requires a change in the electronic spin [4]. Although this kind of transition has a much lower probability to occur than spin-allowed transitions [3,4] the time scale of ISC is similar to the one for fluorescence (10^{-8} - 10^{-7} s) and therefore it competes with fluorescence for the deactivation of S_1. From S_1, the molecule can then pass to the excited triplet state manifold (T_n) and reach, by a succession of VR and IC processes, the lowest vibrational level of T_1. From T_1, and through ISC, the molecule can revert back to the excited singlet state manifold. Since the triplet states have lower energy than the corresponding singlet states, the transition from T_1 to S_1 requires some additional activation energy. This activation energy can be obtained either by a thermal process or by the interaction of two molecules in the triplet state to produce one molecule in the excited singlet state [4,5]. When the molecule returns to S_0 by the emission of a photon, an identical spectrum to the one of conventional fluorescence is obtained [4,5]. This process, which is not represented in the diagram of Figure 1.1, receives the name of delayed fluorescence and has a lifetime of approximately 10^{-3} s.

If reverse ISC does not occur, the molecule has two additional possibilities to return from T_1 to S_0. Through ISC followed by VR or through the emission of radiation in a process called phosphorescence (F). The emission of phosphorescence involves states of different multiplicity and, as a consequence, has a longer lifetime than fluorescence (between 10^{-3} s to 10 s). Since the energy gap between T_1 and S_0 is usually smaller than the one existent between S_1 and S_0, phosphorescence occurs in a region of lower energy than fluorescence.

The detection of phosphorescence is the basis of the analytical technique known as phosphorimetry, which has been widely applied to the

analysis of compounds presenting phosphorescence emission. Phosphorescent compounds usually consist of molecules with extensive π electron systems. These include a large variety of aromatic molecules either with or without heteroatoms in the conjugated system [1-4]. The free movement of π electrons throughout the delocalized molecular orbitals facilitates the electronic transitions responsible for phosphorescence. For molecules with on heteroatoms in the aromatic system, transitions frequently involve the promotion of an electron from a bonding π orbital to an antibonding π* orbital (π→π* transition). Aliphatic molecules, on the other hand, rarely exhibit phosphorescence since the high energy required for π→π* transitions usually causes decomposition prior to excitation [1-4].

Quantum Yield

The intensity of the phosphorescent signal emitted by a compound is determined by its phosphorescence quantum efficiency or phosphorescence quantum yield (ϕ_p) [7,8]. This intrinsic molecule parameter is defined as the ratio between the number of phosphorescence photons and absorbed photons. If such absorbed photon produces one phosphorescence photon, ϕ_p reaches its maximum value (unity). Most times, however, there are several deactivation processes that compete with phosphorescence and result in a ϕ_p value lower than one. Their effect on the phosphorescence quantum yield is best described in terms of their rate constants. Equation 1 [9] relates ϕ_p with the rate constants of fluorescence (k_{FM}), ISC from S_1 to T_1 (k_{ST}), ISC from T_1 to S_0 (k_{TS}), IC from S_1 to S_0 (k_{SD}) and phosphorescence (k_{PT}).

$$\phi_p = \frac{K_{FM}}{K_{FM} + K_{ID} + K_{SD}} \left[\frac{K_{ST}}{K_{PT} + K_{TS}}\right] \qquad (1)$$

In liquid solutions and gas phase, most of the phosphors have very low ϕ_p values as a consequence of the relative high value of k_{PT}. By using high viscosity solvents, low-temperature rigid matrices, or solid substrates, the ISC responsible for the transition from T_1 to S_0 can be

minimized and, therefore, the emission of phosphorescence can be increased.

Lifetime of a Phosphor

The phosphorescence lifetime of a phosphor is defined as the time required for the emission to decrease to 1/e of its original intensity [1,4]. If $I_p(t)$ is the phosphorescence intensity at time t and I_p° is the intensity at $t = 0$, τ_p will be given by

$$I_p(t) = I_p^\circ \exp\left(-\frac{t}{\tau_p}\right) \qquad (2)$$

It is important to distinguish between observed lifetime (τ_p) and intrinsic natural lifetime (τ_p°) of the phosphor. Observed lifetimes are those obtained under a set of experimental conditions such as solvent temperature, environment, and physical state of the sample matrix. These experimental parameters affect the radiationless processes competing with phosphorescence for the deactivation of T_1 and determine to some extent the value of τ_p. The intrinsic natural lifetime can only be measured in the absence of quenching processes, that is, when $\phi_p = 1$. Since radiationless deactivations are usually present in experimental situations, τ_p° cannot be directly determined. However, if the values of τ_p and ϕ_p are known, the intrinsic lifetime τ_p° can be obtained [4] by equation 3.

$$\tau_p = \tau_p^\circ \phi_p \qquad (3)$$

Organic phosphors exhibit considerably greater differences in lifetimes than in either their excitation or emission spectra [10-12]. Keirs et al. [13] were the first to show that mixtures of phosphors with overlapped spectra could be resolved by time resolution. This method was based on the selection of the proper period of time between the cut off of the excitation of the sample and the detection of phosphorescence in such a way that only the emission from the longer lived compound was detected. Since then several instrumental modifications have expanded the scope of

the method improving the selectivity of phosphorimetry and creating a new technique with the name of time-resolved phosphorimetry [4]

The Heavy Atom Effect

The emission of phosphorescence can be enhanced by the presence of atoms of high atomic number (called heavy atom effect) in the environment of the phosphor [2-4] The heavy atom (HA) can be either chemically attached to the analyte (internal HA effect) or introduced in the sample matrix (external HA effect) Between these two effects, the external HA perturbation has been the most used method to improve the sensitivity of phosphorimetry

The enhancement of the phosphorescent signal by the presence of HA in the environment of the phosphor is the result of an increase in the rates of spin-forbidden processes This is a phenomenon called spin orbit coupling interaction The orbital motion of the electron involves a magnetic field that interacts with its spin magnetic momentus changing the direction of the electronic spin [2-4] As a consequence of the enhancement of the singlet-triplet ISC rate there is a decrease in the fluorescence quantum yield which results in a decrease in the fluorescence intensity [14] Since the presence of HA can also increase the radiationless rates of triplet state decay to the ground singlet state [4] the phosphorescence quantum yield is not always increased and the opposite effect has also been noted [14 15] Most times however the phosphorescence decay is more strongly enhanced In addition the external heavy atom perturbation causes a decrease in the phosphorescence lifetime of the phosphors which also reduces the probability of radiationless deactivation of the triplet state by collisions [2-4]

Phosphorimetry as an Analytical Technique

As a means of chemical analysis phosphorimetry was first suggested in 1937 by Kelra and co-workers [13] Since then the technique has grown to become an important tool in the trace analysis of compounds presenting phosphorescence emission [1-4]

According to the experimental procedure phosphorimetry can be divided in four different techniques, which include solution low-temperature phosphorimetry (LTP), solid-surface room-temperature phosphorimetry (SSRTP), micelle-stabilized room-temperature phosphorimetry (MS-RTP) and sensitized room temperature phosphorimetry in liquid solutions (sensitized-RTPL).

In the four techniques most analyzed compounds have shown to some extent a linear relationship between the intensity of the phosphorescence emission and the analyte concentration.

For optically homogeneous and perfectly transparent solutions as the ones frequently tested in LTP, sensitized RTPL and certain micellar cases the intensity of the phosphorescence emission (I_p) is related to the intensity of the excitation light (I_o) by the following equation [1 4]

$$I_p = \phi_p (I_o - I_T) \tag{4}$$

where I_p is the intensity of the transmitted light. For transparent media I_T can be obtained from the Lambert Law [4], which is given by

$$I_T = I_o \times 10^{-\epsilon b c} \tag{5}$$

where ϵ is the molar absorptivity, c is the analyte concentration and b is the thickness of the sample. Equation 4 can then be re written as

$$I_p = I_o \phi_p (1 - 10^{-\epsilon b c}) \tag{6}$$

This expression can be expanded in a Taylor series to yield

$$I_p = I_o \phi_p [2.303 \epsilon b c - \frac{(2.303 \epsilon b c)^2}{2!} + \ldots] \tag{7}$$

For very dilute solutions $\epsilon bc \ll 1$ and all but the first term in the series expansion can be dropped. The resultant expression is [4]

$$I_p = 2.303\phi_f I_o \varepsilon b c = Kc \qquad (8)$$

which shows the direct proportionality between I_p and c

For optically inhomogeneous and non-transparent media the applicability of Lambert Law has severe limitations [1 4] In RTP measurements performed on solid substrates (SSRTP) or snow-like matrices (certain LTP cases), both excitation and emission radiation are extensively scattered In addition, the excitation radiation can be absorbed by the solid matrix as well as the analyte Any theoretical model employed to derive an expression relating intensity and concentration must take these effects into consideration

Kubelka and Munk proposed a set of differential equations of the first order in which the complex process of light propagation inside inhomogeneous scattering media is characterized by only two coefficients [16,17] Although this model is based on several restrictive assumptions that are not frequently fulfilled in experimental situations, its simplicity has allowed spectroscopists to develop the foundations for most quantitative studies [4] Zweidinger and Winsfordner [17] utilized a modification of the Kubelka-Munk differential equations to obtain an expression for the phosphorescence intensity emitted in snow like matrices and cracked glasses At low concentrations of analyte the intensity of phosphorescence is given by

$$I_p = 2\phi_f I_o K\bar{b}\left[\frac{1}{1+2s\bar{b}}\right] = 2\phi_f I_o \frac{2.3\varepsilon\bar{b}}{(1+2s\bar{b})}c \qquad (9)$$

where

k = fraction of radiation absorbed per average path length
(k = 2.303 ε)

$\bar{b}$ = average cell path length for diffuse reflectance cm^{-1} and

s = fraction of exciting radiation scattered per path length cm^{-1}
(s = function of analyte concentration)

if the scattered light is negligible ($s\bar{b} \ll 1$) equation 9 reduces to

$$I_p = 2\phi_p I_o \bar{a}\bar{b} = 4.6\phi_p I_o \varepsilon b c \qquad (10)$$

At high analyte concentrations the intensity of phosphorescence emission is given by

$$I_p = 2\phi_p I_o \left(\frac{\sqrt{k}}{\sqrt{k+2s} + \sqrt{k}}\right) \qquad (11)$$

if a >> k equation 11 reduces to

$$I_p = 2\phi_p I_o \sqrt{\frac{k}{2s}} \qquad (12)$$

If k >> s I_p is then given by

$$I_p = \phi_p I_o \qquad (13)$$

Equations 10 and 13 show that the analytical curves (log I_p vs log c) in inhomogeneous diffusely reflecting media should have a slope of unity and zero at low and high concentrations respectively These theoretical expressions were successfully supported by experimental results obtained at low temperature [17] In most cases however the same behavior has been observed in SSRTP where the diffusing and reflecting media consists of solid substrates [4]

Until 1975 most work involving phosphorescence was carried out at low temperature (77°K) By the use of cryogenic equipment the solutions were frozen to form a rigid matrix to minimize the collisions competing with phosphorescence for the deactivation of the triplet state Since then very little has been done in the area of LTP The lack of widespread use can be attributed to three major reasons [15 19] The high cost of cryogenic equipment and liquid coolant the time consuming deoxygenation of the solvent to avoid quenching effects and the inconvenient procedure to introduce the sample into the cooling system Low limits of detection (LOD) (nanomolar range) calibration curves with wide linear dynamic ranges (LDR) (10^4 10^5 fold change in analyte concentrations) and relative standard deviations (RSD) of measurements

ranging from 1 to 5% were only obtained with clear glasses matrices. Variations in the rate of sample heating caused modifications in the final matrix, resulting either in a cracked glass or in a snow. In addition, the cell positioning had to be carefully performed to reach good reproducibilities. In such a way that the presence of a skilled technician or chemist was necessary to obtain satisfactory results. These problems turned LTP into an inconvenient tool for routine analysis of a large number of samples.

Around 1972 to 1973, Schulman and Walling [20,21] reported the observation of strong phosphorescence emission from a wide variety of ionic organic compounds adsorbed on solid substrates. Silica, alumina, paper, asbestos, and glass fiber were employed to provide the rigidity required to restrict collisional quenching and to prevent radiationless deactivation of the triplet state. In contrast to LTP, the sampling procedure was very simple and required no more than 10 min per sample. Following these early reports, a large number of studies dealing with the optimization of parameters affecting the sensitivity and reproducibility of SSRTP have appeared in the literature. Depending on the analyte, solid surface, and experimental conditions, calibration curves with LDR of 10^2 10^3 fold change in analyte concentration, LOD in the subnanogram or nanogram region, and RSD ranging from 5 to 15% can be easily obtained. The simplicity of the sampling procedure, elimination of the need for cryogenic equipment, and the possibility of automation have turned SSRTP into the most popular phosphorimetry technique of the last two decades [18].

Micelle-stabilized room-temperature phosphorimetry in aqueous solution [22-24] was first applied as an analytical method of analysis by Cline Love and co-workers [25,26]. The method was employed to determine a wide variety of phosphors including ketones, aldehydes, alcohols, carboxylic acids, phenols, amines, and compounds of pharmaceutical interest. Micellar solutions consist of colloidal aggregates of surfactants or detergents which protect the phosphorescing triplet state

of the analytes from external quenchers and vibrational deactivation. The aggregation of surfactants only occurs above a certain concentration known as critical micelle concentration (CMC). This concentration depends upon the chemical structure of the surfactant and the nature of the solvent. In order to obtain satisfactory results, the CMC value of a particular surfactant has to be determined before its use. If no data are available in the literature, the CMC determination can be performed either by tensiometry or by fluorescence direction of a probe compound [25 26]. The main disadvantage of the technique, however, is the necessity of thorough sample deoxygenation, which results in a sample analysis time of approximately one hour, restricting the use of MS-RTP in routine analysis of a large number of samples [4]. There are, nevertheless, considerable advantages in the use of the micellar method. In most cases, the sensitivity and precision of MS-RTP are comparable to those obtained by LTP [18]. The RTP analysis of large multicyclic aromatic compounds in aqueous solutions is facilitated by the high solubility of this species in the micellar environment, and finally, the micelle approach offers a means to conduct phosphorescence analysis that can be directly applied to flow systems and liquid chromatography detection [4].

RTP detection in continuous flows and chromatographic systems can also be performed by means of immobilized RTPL [27 29]. In this technique, the weakly or non phosphorescent analyte (donor) transfers its triplet energy to a given acceptor. A suitable acceptor should have a triplet energy lower than that of the donor, a high quantum phosphorescence yield and a lower molar absorption in the excitation region of the donor. In addition, the energy gap between the triplet states of the donor and the acceptor should be enough to prevent significant reverse mechanisms. So far, the most appropriate acceptor tested for the technique has been bisacetyl (BIAC) [28]. LOD in the nanomolar range were obtained by flow injection of several polyhalogenated naphthalenes and biphenyls and liquid chromatography analysis of mixtures of chlorinated naphthalenes, biphenyls, and dibenzofurans [29]. According to the authors [28 29]

several advantages justify the use of sensitized RTPL as a detection technique. Even though sensitized RTPL is based on an emission technique, it is inherently more sensitive than UV-absorption; the background noise due to scattering and fluorescing impurities is negligible at the emission wavelength of BiAC (522 nm), and it is particularly selective since all of the constituents of a sample do not produce the sensitized phosphorescence signal of the acceptor. Further research, however, is necessary to develop new acceptor systems and extend the application potential of sensitized RTPL to other kinds of compounds.

CHAPTER 2

EXPERIMENTAL CONSIDERATIONS FOR SOLID SUBSTRATE ROOM TEMPERATURE PHOSPHORIMETRY

Even though Kath [30] was the first to report the use of cellulose as a rigid matrix to enhance the phosphorescence emission of several compounds at room temperature, the potential of SSRTP as an analytical technique was only recognized after the studies performed by Schulman and Walling [20,21]. When adsorbed on solid substrates such as paper, silica, alumina, asbestos and glass fiber, the ionic forms of salts of a wide variety of polynuclear carboxylic or sulfonic acids, phenols, and amines showed comparable phosphorescence intensities to those emitted by the same compounds at low temperature [20,21]. At room temperature, the strongest signals were obtained on filter paper. The sampling procedure was very simple. Prior to signal detection, Whatman No. 1 filter paper strips were saturated with the analyte solution and slowly dried under an infrared lamp to avoid the presence of humidity, which showed to be a quencher of the phosphorescence signal. The presence of oxygen, which is a serious phosphorescence quencher in solution measurements, did not show significant effect on the RTP signal of most studied compounds. The fact that no phosphorescence emission was detected from finely ground samples of pure salts or from the corresponding crystals grown from solutions of the organic acids led the authors to conclude that the matrix rigidity necessary to the emission of strong phosphorescence was obtained via absorption of the emissions to the surface of the substrate [20,21]. Since no room temperature phosphorescence was observed from nonionic compounds, it was also suggested that the ionic state of the molecule increased the rigidity of the matrix and reduced, as a consequence, the collisional radiationless deactivation of the triplet state [20,21].

The first application towards the analytical usefulness of the phenomenon was reported by Winefordner and co-workers [31,32]. Great care was taken in developing a rapid and reproducible method to determine small amounts of acid derivatives of naphthalene [31] and compounds of biological interest [32]. By using a microliter syringe, 5 µl of a 1M NaOH solution of the desired analyte were spotted on Eaton Dikeman 613 filter paper discs. Several drying methods including oven, hot air blower, air drying followed by a period in a desiccator, and infrared lamp were searched for best results. In the analysis of acid derivatives of naphthalene [31], the most reproducible and strong signals were obtained by air drying followed by a period in a desiccator. This procedure, however, was time consuming and involved more sample handling than desired. Heating the sample by means of an oven or a hot air blower proved to be too destructive for the analyte and the substrate [31,32]. Infrared lamp, though, showed to be both gentle and effective [32]. LOD in the nanogram level and calibration curves between one and four orders of magnitude were easily obtained in an analysis time of approximately one minute per sample [32]. In agreement with the original assumption that the ionic strength of the phosphor was directly related to the induction of RTP on solid substrates [2D 21], emission of phosphorescence from nonionizable compounds such as polyaromatic hydrocarbons (PAHs) was not observed [32]. Relatively strong phosphorescence, however, was detected from some uncharged species such as sulfoguanidine and 5-acetyl-uracil. According to the authors [32], this anomaly was indicative that binding forces other than ionic interactions were involved in the induction of SSRTP. Hydrogen bonding between the phosphor and the hydroxyl groups of the filter paper was then suggested as one of the possible mechanisms to avoid radiationless deactivation of the triplet state [32].

In order to further investigate the nature of the support-phosphor interaction which results in the observation of RTP, Schulman and Parker [33] studied the phosphorescence signal of 4-biphenylcarboxylate and sodium 1-naphthoate at room temperature. The analytes were spotted on

several supports of different polarity. Strong visible phosphorescence was observed from all substrates containing an abundance of surface hydroxyl groups such as paper, sucrose, starch, and 50% SWP polyethylene fiber - 50% paper blend. No appreciable phosphorescence, however, was noted from supports containing few or no surface hydroxyl groups such as SWP polyethylene fiber, borosilicate glass fiber, paper, or stainless steel. In addition, a reduction of 94% in intensity was noted when the phosphorescence signal of 1-naphthaste spotted on treated Whatman No. 1 filter paper was compared to the one emitted by the same compound spotted on filter paper with no treatment. The treatment, which was performed by the immersion of the filter paper in dimethyldichlorosilane, removed most of the hydroxyl groups from the surface of the substrate. According to Schuleen and Parker [33], the requirements of a polar or ionic compound combined with the necessity of a support rich in hydroxyl groups strongly indicated hydrogen bonding as the primary mechanism of inducing RTP emission on solid substrates. Supporting evidence for this kind of interaction was also provided by von Wandruszka and Hurtubise [34, 35]. In the search for a new substrate for SSRTP, they observed strong phosphorescence emission from p-aminobenzoic acid (PABA) adsorbed on sodium acetate (NaOAc) [34]. The mode of adsorption of PABA on the substrate was then investigated by infrared (IR) spectroscopy [35]. By comparing the IR spectra of PABA, NaOAc, and PABA adsorbed on the substrate, it was noted that the N-H stretching vibrations of PABA at 3350-3450 cm^{-1} disappeared in the spectrum of the phosphor adsorbed on NaOAc. This fact was attributed to hydrogen bonding between the amino group of PABA and the carboxyl groups of NaOAc. The N-H vibrations were probably shifted to a spectral region of longer wavelengths and overlapped by the broad methyl stretch band of NaOAc at 3000 cm^{-1} [35]. Additional hydrogen bonding evidence was later reported in SSRTF studies involving a large variety of nitrogen heterocyclics and aromatic amines adsorbed on silica gel and polyacrylic acid/salt mixtures [36-41]. Both kinds of substrates contain active sites for potential hydrogen-bond formation

The analytes were spotted from neutral, acidic and basic solutions onto each support and the relative phosphorescence intensities were compared. In general, analytes spotted from acidic solutions produced the greatest signal intensities. Their IR spectra confirmed the formation of hydrogen bonds between compounds and substrates. Strong phosphorescence emission, however, was also detected from compounds incapable of forming hydrogen bonds with the adsorbent [39-42], suggesting that other mechanisms than hydrogen bonding are involved in the emission of phosphorescence at room temperature.

For the specific case of compounds adsorbed on cellulose paper, a matrix isolation mechanism for RTP induction has been recently proposed [43]. The mechanism is based on inherent properties of the substrate. Cellulose undergoes considerable swelling in the presence of polar agents such as water and ethanol, which are the most common solvents employed in SSRTP analysis. The swelling opens up new areas in the paper fibers, facilitating the entry of phosphor molecules into submicroscopic pores. When the substrate is dried, the cellulose fibers collapse and the phosphor molecules become trapped between the cellulose chains. As a consequence, the molecular vibrational motions responsible for radiationless deactivation of the triplet state are restricted; the phosphor is protected from quenching by molecular oxygen, and an enhancement in the RTP signal is observed [43]. This mechanism was evaluated by studying the behavior of the phosphorescence signal of 4-biphenylcarboxylic acid (4-BPCA) and o-aminobenzamide (oABZA) adsorbed on Whatman No. 3 MM chromatography paper. Prior to analyte deposition, the substrate was spotted with solvents of different polarity such as benzene, acetone, propanol, ethanol, methanol, and water. Benzene and acetone did not swell the cellulose fibers, but the other four solvents swell the substrate in increasing order from propanol to water. It was observed that the 4BPCA samples exhibited no appreciable intensity when prepared in nonswelling media. On the other hand, the RTP intensities of 4BPCA samples prepared with the polar solvents were significant and increased in

the exact order of matrix swelling The same trend was observed for the eASZA samples except that the phosphor displayed significant intensity when prepared with benzene or acetone The polar solvents though promoted much higher RTP signals than the nonpolar media Further information was provided by comparing the phosphorescence intensities of monocyclic and bicyclic compounds adsorbed on cellulose previously spotted with water [43] Since small molecules supposedly penetrate cellulose pores easier than large ones, stronger phosphorescence emission was expected from monocyclic phosphors The expectations were confirmed, suggesting that not only cellulose swelling but also steric factors play an important role in the immobilization of phosphor molecules on paper substrates [43] It is important to mention however that the comparison between the phosphorescence signals of monocyclic and bicyclic compounds was made without taking into consideration the phosphorescence quantum yields of the studied compounds

Although a great deal of effort has been focussed on understanding the mechanism responsible for the emission of room temperature phosphorescence on solid substrates, it has not yet been possible yet to provide a logical foundation for the development of new RTP methodology To date, the selection of appropriate substrates and best experimental conditions for SSRTP detection has been based on information obtained from previous analysis If data are not available in the literature the optimization of parameters is usually performed on a trial and error basis which involves time consuming procedures Once the method is developed for a particular analysis though, phosphorescence data are fast and simple to acquire Simplicity of experimental procedure, sensitivity selectivity, small sample requirements and low cost are advantages that have turned the technique into an attractive tool for the analysis of compounds of diversified interest [7 44]

Quenching Effects

The phosphorescence intensity emitted by compounds adsorbed on solid substrates may be decreased or even eliminated by interactions with other

chemical species [2] One of the most efficient triplet quenchers is molecular oxygen [4] Interaction of triplet state oxygen (the natural ground state of oxygen) with a molecule in an excited triplet state results in the radiationless deactivation of the excited molecule and the production of excited singlet state oxygen [7] Although the presence of oxygen in SSRTP is not as critical as it is in RTP solution measurements [2,4], it can cause to some degree quenching of phosphorescence emission Schulman and Parker [33] studied the oxygen effect in the phosphorescence signal of NaNPCA adsorbed on filter paper disks A reduction of approximately 30% in emission intensity was noted when the phosphorescence signals of samples exposed to dry oxygen for 25 minutes were compared to those measured under a flow of dry argon Va-Dinh and co workers [45] observed a reduction of 50% in the phosphorescence emission of pyrene when the intensity measured under an oxygen atmosphere was compared to the one detected under a flow of dry nitrogen Similar behavior was noted by Kasar and Winefordner [46] in the study of several polycyclic hydrocarbons Reductions ranging from 50% (pyrene) to 75% (phenanthrene) were verified when the RTP emission intensities of samples measured under dry air were compared to those obtained under nitrogen or argon atmospheres Such strong oxygen quenching effects however were not reported for highly polar compounds When PABA was spotted on filter paper and subjected to dry air, argon and nitrogen flows, similar phosphorescence intensities were observed [46] Strong RTP emission was still detected from phthalic acid isomers, PABA and terephthalamide adsorbed on silica gel surfaces and measured under a flow of dry air [36] The presence of oxygen in dry air however, quenched the RTP emission of nitrogen heterocycles adsorbed on silica gel and filter paper [47] Furthermore, it was noted that the RTP of compounds adsorbed on silica gel was generally more subject to oxygen quenching than the RTP of the same compounds adsorbed on filter paper The percent enhancement of benzo[f]quinoline measured in helium versus air for example was 53% for silica gel and only 16% for filter paper According to the authors [47]

this seemed reasonable since silica gel is more porous than filter paper and therefore has more surface area on which oxygen could potentially interact with adsorbed compounds. The influence of oxygen transport into sample matrices used in SSRTF was also studied by Hilday and Seybold [48]. The phosphorescence lifetime of 2-naphthalene sulfonate adsorbed on filter paper was increased by treating the substrate with substances such as CH_3COONa, H_3BO_3, NaF, NH_4Cl, glycine, glucose, and sucrase. The RTF intensity was enhanced by the presence of sucrose, but the effect of other substances was less predictable and needed further investigation. The authors [48] suggested that the added substances could be plugging up the channels and interstices of the matrix, decreasing the permeability to oxygen. In addition, these materials could impart a rigidity to the substrate and inhibit specific internal motions responsible for radiationless deactivations of the triplet state. An opposite effect to the one observed by Hilday and Seybold [48] was obtained when RTF measurements were performed under humid conditions. Using paper as a sample matrix, Schulman and Parker [33] demonstrated that oxygen quenching occurs much more freely in the presence of moisture. The efficiency of oxygen quenching in the RTF emission of NaBPCA increased dramatically as the relative humidity of the oxygen atmosphere increased. The authors [33] suggested that moisture could be disrupting hydrogen bonds between phosphors and substrate, facilitating the transport of oxygen into the vicinity of the phosphor. The presence of moisture itself has proved to be an extremely powerful quenching agent of RTF emissions [20, 21, 31-33, 36]. Besides facilitating the interaction between oxygen and analyte, water molecules might compete with the phosphor for bonding sites on the support, thus decreasing the analyte-support adsorption process and increasing the probability of collisional and vibrational quenching. For maximum RTF intensity, therefore, it is recommended to dry the sample prior to detection and keep it dry during the measurements. Several methods have been employed in the drying step [4]. One of the most common is the use of an infrared heating lamp. This method is rapid, convenient

and generally nondestructive for both the analyte and the substrate. Between 3 and 5 minutes are required for dry filter paper samples, and the drying temperature can be set by adjusting the distance between the sample and the lamp. The presence of moisture during the measurements can be easily avoided by passing a gentle flow of dry nitrogen or dry argon through the sample compartment of the spectrophosphorimeter. The inert gas is usually dried by passing it through one or two drying cartridges filled with calcium sulfate and placed prior to the inlet of the spectrophosphorimeter.

Background Influence on SSRTP

The sensitivity of an analytical method is usually evaluated in terms of limit of detection (LOD). The value of the LOD is directly related to the smallest measure that can be detected with reasonable certainty [49]. According to the International Union of Pure and Applied Chemistry (IUPAC), the smallest discernable signal (x_L) is given by the following expression:

$$x_L = \bar{x}_b + ks_b \qquad (14)$$

where $\bar{x}_b$ is the main average of the blank responses, s_b is the standard deviation of the blank measurements, and k is a numerical factor chosen in accordance to the confidence level desired [49]. From equation 14, it can be seen that the lowest x_L values (and s_b) are obtained for the lowest signals of analytical blanks. Therefore, in order to obtain the highest detection power with a specific analytical method, it is convenient to reduce the blank signal to the minimum possible value.

In SSRTP measurements of solutions of pure compounds, the analytical blank signals are obtained by measuring the phosphorescence emission of solvents spotted on solid substrates. In most SSRTP determinations, relatively pure solvents are used and the main contribution to the phosphorescence background is usually given by the solid substrate. As with other analytical methods, low SSRTP detection limits are obtained with low background signals. As a consequence, many materials have been searched to find a substrate with low phosphorescence emission between 350

and 650 nm, which is the spectral region where most compounds phosphoresce. In addition, a suitable SSRTF substrate should present the versatility to enhance, as much as possible, the RTP signal of a wide variety of phosphors with different physico-chemical characteristics. Among the materials investigated cellulose and particularly filter paper appears to be the most convenient substrate for the technique [4, 12, 15, 32]. The main advantages of cellulose over other materials are low cost, simplicity of handling and availability of a wide selection of different papers enabling one to select the best substrate for phosphors with diverse characteristics. Nevertheless several problems are related to its use in SSRTF. Sensitivity to oxygen and moisture, nonuniformity in the surface and variation in the physical and chemical composition within the same batch can deteriorate the reproducibility and sensitivity of measurements [4]. However the major disadvantage is the broad featureless bands of excitation spectra from 200 to 400 nm and phosphorescence spectra from 375 to 625 nm [43]. This background phosphorescence emission which is often of the same order of intensity as the analyte signals from relatively high analyte concentrations severely limits the sensitivity of SSRTF [50]. Lignin, hemicelluloses, or even transition metal traces have been suggested as possible sources of the background signal [51]. Several attempts have been made to decrease this signal, to some extent, using solvent extraction or chromatography, chemical or heat treatment, or signal background subtraction with the help of a computer assisted spectrophosphorimeter, by Winefordner and re workers [50-52]. The most significant reductions however were obtained by ultraviolet irradiation treatments [53, 54]. McAleese and Dunlop [53] observed a reduction of 95% in the phosphorescence emission of filter paper irradiated at 265 nm for 57 hours in the sample compartment of the spectrophosphorimeter. The time of ultraviolet treatment was later reduced in a modification proposed by Campiglia and DeLima [54]. Strips of filter or chromatography paper were water extracted in a soxhlet apparatus for 8 hours, introduced into quartz tubes and exposed to

ultraviolet radiation for the same period of time. The ultraviolet treatment was carried out in a photochemical reactor equipped with five lamps with λ_{max} emission at 254 nm and seven with λ_{max} emission at 300 nm. A 94% background reduction was obtained, which allowed the picogram detection limits for PABA, carbaryl (1-naphthyl N-methylcarbamate) and 1-naphthol to be obtained. Since the low background signal remained constant for several weeks after treatment, the strips of paper could be stored in a desiccator to be conveniently used in routine analyses.

External Heavy Atom Effect

The sensitivity of an analytical method can be improved not only by reducing the intensity of the analytical blank but also by increasing the analyte signal. As discussed in Chapter I, the phosphorescence emission of compounds adsorbed on solid substrates can be greatly enhanced by the presence of heavy-atoms in the sample matrix. Seybold and White were the first to report on the external heavy-atom effect in SSRTP [55]. They observed an increase of about 40 times in the phosphorescence intensity of 2-naphthalene sulfonate when 1.0 M KI was spotted on the filter paper containing the analyte. Similar results were obtained for 1-naphthalene sulfonate, 2,7-naphthalene disulfonic acid, 1-naphthol, 2-naphthol, 2-naphthol-6-sulfonate and 2-naphthol-7-sulfonate. Vo-Dinh and co-workers extended the use of iodide as an external heavy-atom perturber to the SSRTP analysis of a wide variety of compounds of biological and pharmaceutical interest [56]. The analyte solutions were prepared in 1M NaOH-1M NaI (1:1), spotted onto filter paper discs and dried for about 30 minutes by infrared lamp heating. The heavy-atom enhancement factor, which was defined as the ratio of the analyte phosphorescence signal in 1M NaOH-1M NaI (1:1) solution to that in 1M NaOH aqueous solution, ranged from 1 for 6-chloropurine to 12 for guanine, and 40 for tryptophan. In some cases the LOD were reduced by one or two orders of magnitude, showing the advantage of using iodide to improve the sensitivity of SSRTP. Comparable enhancement factors, though, were not observed when NaI was employed in the analysis of quinoid compounds. In the study of the phosphorescence

behavior of anthracene pyrene phenanthrene, benzo[e]pyrene and coronene. Vo-Dinh and co-workers [57] stated that the addition of NaI to basic solutions of these compounds induced only weak phosphorescence signals. The use of silver nitrate however considerably enhanced the phosphorescence intensities of neutral solutions of phenanthrene and pyrene. For these two phosphors, LOD in the nanogram level were obtained, opening up new possibilities for the SSRTP analysis of nonpolar compounds. The effect of a variety of heavy-atom salts on the RTP signal of cinoxicin was later reported by Jakovljevic [58]. Solutions of salts of thallium, lead, thorium, europium, etc., were employed to soak filter paper strips prior to cinoxicin deposition. After drying, the substrate for 10-15 seconds with an air blower at room temperature, one microliter of analyte solution was spotted on the filter paper strips and dried for 10 minutes in an oven at 105°C. The highest enhancements were obtained with lead tetraacetate, thallous fluoride and thallous acetate. By employing a 0.5% or 1% solution of lead tetraacetate, amounts of cinoxicin as low as 50 pg were successfully measured, and calibration curves with LDR between 0.002 - 0.1 μg were obtained. Lead (II) and thallium (I) were also employed to enhance the phosphorescence emission of PAHs adsorbed on filter paper. Lue-Yen and Winefordner [46] selected $AgNO_3$, $Pb(NO_3)_2$, $HgCl_2$, $Hg(NO_3)_2$, and $CH_3CH_2O_2Tl$ to study the heavy-atom effect on the phosphorescence emission of chrysene, coronene, 1,2:5,6-dibenzanthracene, fluoranthrene, and pyrene. Of the heavy ions, thallium (I) seemed to be the greatest enhancer of RTP signals for all PAHs with the trend being $Tl^+ > Ag^+ > Pb^{2+} > Hg^{2+}$. Based on the enhancements obtained, the authors [46] estimated that the LOD of the studied compounds could be lowered up to 6-fold by using thallium (I) salt. Not only the sensitivity but also the selectivity of SSRTP can be improved with the presence of heavy atoms in the sample matrix. Vo-Dinh and Hooyman [59] proposed the use of different heavy-atom salts to determine target compounds in complex mixtures without previous separation. The new approach, which was called selective external heavy-atom perturbation (SEHAP), was based on the fact that preferential

enhancement by certain heavy-atoms of some phosphor signals occurred much more than for others. SEHAP was applied to the qualitative and quantitative analysis of several mixtures (up to seven components) of polynuclear aromatic compounds of environmental and biological importance. Among the heavy atom salts tested, the highest RTP enhancements were obtained with $AgNO_3$, CsI, NaI, NaBr, $Pb(CH_3CH_2O_2)_2$ and $LiClO_4$. Since their concentrations could have an effect in the RTP enhancement factors, a previous determination of their optimum values for maximum RTP emission was performed by measuring the phosphor-atom signal of analyte solutions at several heavy-atom concentrations. Three distinguishable ranges were observed. At very low concentrations (less than 10^{-2} M), the amounts of heavy atoms present in the sample spot were insufficient to induce significant phosphorescence enhancement, at medium concentrations (more than 10^{-2} M) RTP increased with heavy-atom concentrations, at high concentrations some saturation effects occurred and the increase in heavy atom amounts no longer caused any substantial increase in the RTP signal. Saturation though did not depend only upon the absolute concentration of heavy atoms but also upon the relative ratio between heavy-atoms and analytes. The saturation condition which corresponded to the maximum RTP enhancement was achieved at high heavy-atom concentrations and sufficiently low analyte concentrations. Since most heavy-atoms were near their solubility limits at 0.5-2 M concentration range, analyte concentrations lower than 10^{-3} M could be measured to satisfy the requirement for saturation. This fact, however did not represent any limitations for analytical purposes because most RTP measurements deal with sample solutions within 10^{-5} - 10^{-8} M ranges. The highest phosphorescence enhancements were obtained with 0.5 M $AgNO_3$, 0.2 M CsI, 2 M NaI, 2 M NaBr, 0.5 M $Pb(CH_3CH_2O_2)$ and 2 M $LiClO_4$. By selecting the appropriate combination of heavy atom solution and excitation wavelengths the authors [59] were able to perform the analysis of specific target compounds with practically no interferences from the other components of the mixture. Most compounds measured were in the

nanogram level range showing the potential of SSHAF as a useful tool in the SSRTF analysis of mixtures without previous separation The external heavy atom effect has also been applied to the SSRTF determination of several pharmaceutical compounds [60 62] pteridines [63], indoles [64], and pesticides [54 65 67] In most cases the presence of heavy atoms has successfully improved the sensitivity of the technique allowing a LOD to be obtained in the nanogram-picogram range

The effectiveness of heavy atom perturbers can be increased by the presence of surfactants in the matrix of the phosphor DeLima and co-workers [68] were the first to demonstrate the advantage of using surfactants on SSRTP They studied the effects of thallium and silver nitrate, thallium and silver dodecyl sulfates (TlDS, AgDS) potassium iodide and sodium dodecyl sulfate (NaDS) on the phosphorescence intensity of carbaryl Three µL of analyte ethanolic solution (20 µg/mL) were spotted onto filter paper discs dried under a stream of nitrogen for 20 minutes and their RTP signals assessed (I_o) Subsequently, 3 µL of the heavy atom enhancer solution were added to the same spot and the drying and assessing procedures were repeated (I_e) The enhancement factors (I_e/I_o) varied from 1-5 to 20 depending on the type and amount of enhancer used Although the NaDS molecule does not contain a heavy-atom a 1-5-fold enhancement factor was observed According to DeLima et al [68] the presence of NaDS on the surface of the paper could provide other sites of intercalation for the phosphor resulting in increased rigidity of the molecule and, therefore, higher intensity of the phosphorescence emission The enhancement factors of the lower concentrated solutions (0 005 M) of TlDS and AgDS were higher than those obtained from similarly concentrated solutions of KI $AgNO_3$ or $TlNO_3$ A comparison of the 0 05 M AgDS and TlDS with their corresponding nitrate salts showed increased intensity in the net phosphorescence signal of 2 7 and 4 8 respectively In addition it was observed that an increase of the concentrations of the surfactant salts on the spotting solution had a marked effect on the intensity of the phosphorescence when the concentrations of AgDS and TlDS were increased

from 0 05 M to 0 02 M, a 2- to 3-fold increase in the intensity of cetheryl was estimated A similar increase of silver and thallium only produced ca 1 2 fold increase in the intensity At 0 02 M concentration the net phosphorescence signals in the presence of the surfactant salts were higher by a factor of 7 0 and 9 5 for silver and thallium respectively In order to obtain a better understanding of the reasons for the observed phenomenon, x-ray photoelectron spectroscopy (XPS) analysis of the sample was performed The XPS data revealed that both TlOS and AgDS were better retained on the surface of the paper than their corresponding nitrate salts According to the authors [68], this was indicative that the observed phenomenon could be attributed to the increased availability of the heavy atom enhancer at the surface of the substrate which probably facilitated the interaction between heavy-atoms and phospher molecules Since the long hydrocarbon chains of the surfactant could avoid the penetration of phospher molecules into the cellulose pores and increase their interaction with heavy-atoms at the surface of the substrate, DeLima et al [68] suggested that higher RTP enhancements could be obtained if AgDS or TlDS solutions were spotted on the paper prior to the phospher or if a premixed solution of the phospher with AgDS or TlDS was utilized The use of surfactants in SSRTP was later extended by Ramis Ramos et al [69] to the analysis of several PAHs and carbazole The influence of the surfactant character on the RTF signal of nonpolar compounds was studied by employing o-terphenyl as a model compound Sodium dodecyl sulfate (SDS) and sodium decyl sulfate (SdS) were selected as anionic surfactants, Brij 35 was the nonionic species and dodecyltrimethylammonium chloride (DTAC) was chosen as the cationic surfactant The highest phosphorescence signals were observed by spotting, on filter paper discs previously treated with 0 25 M $TlNO_3$, a mixture of the analyte dissolved in heptane with an aqueous solution of the surfactant The highest enhancement factors were obtained in the presence of SDS (4 4) and SdS (5 3) A drastic intensity reduction was observed with the other two surfactants In the case of DTAC the

spectrum of the analyte disappeared completely. As a possible explanation, Davis Ramos and co-workers [69] suggested that the higher affinity between the analyte and the nonpolar surfactant tails increased the probability of interaction between phosphor molecules and thallium ions. In addition, association of nonpolar analytes with the surfactants could aid the migration of such species inside the submicroscopic pores of the cellulose fibers to produce a better entrapment of the analyte and reduce radiationless deactivation of the triplet state. By using a 2% SDS solution, all studied compounds showed LOD in the subnanogram level. When their values were compared to those obtained in the absence of the surfactant, an improvement factor of 2–10 X was verified. LOD in the subnanogram level were also obtained for PAHs and phenothiazine derivatives [70]. The analyte solutions containing 3% SDS were spotted on filter paper discs previously treated with 0.1 M $TlNO_3$. Before measurement, the samples were dried for 10 min in an oven at 110 °C and for 15 min in the sample compartment of the spectrofluorimeter under a flow of dry nitrogen. The order of addition of reagents on the filter paper was modified in studies performed by Parry and co-workers [71,72]. A micellar solution of thallium lauryl sulfate (TlLS) was employed in the SSRTP analysis of anthracene [71] and several PAHs [72]. The TlLS solutions were spotted on the paper substrates prior to analyte deposition. In both cases [71,72], it was observed that the highest RTP enhancements were obtained with TlLS concentrations ranging from 0.016 to 0.065 M. For these concentration ranges, the net phosphorescence signal of anthracene with TlLS was double the one observed with $TlNO_3$. For all analytes studied, the LOD were in the nanogram level and compared favorably with those in literature reports, which revealed a variety of filter papers and matrix modifiers. According to the authors [72], these studies suggested that filter paper previously treated with TlLS could be used as a general method for trace analysis of different kinds of PAHs. The substrate was stable over a long period of time, meaning it could be prepared ahead of time and stored for future use in routine analysis.

CHAPTER 3

AUTOMATIC SAMPLING SYSTEM FOR SSRTF ANALYSIS

Introduction

Although SSRTF is a simpler technique to work with than low-temperature phosphorescence, the individual sampling procedure which is usually used to perform SSRTF analysis is time consuming and therefore inconvenient for applications where numerous samples are handled routinely. In addition, the several manual steps involved in sample preparation can lead to appreciable random errors which affect the reproducibility of the technique.

Few attempts have been made to reduce the manual steps and the sampling time involved in SSRTF analysis [45 73]. Vo-Dinh and co-workers [45] designed an automatic phosphorimetric system coupled to an Aminco-Bowman spectrofluorimeter with the sample compartment modified for detection of phosphorescence. A laboratory constructed rotating mirror assembly was employed to eliminate the interference of fluorescence emission and excitation scattered light in the detection of RTF signals. The excitation light beam from the excitation monochrometer was reflected by the mirror onto the surface of the substrate which moved horizontally across the slit located at the top of the sample compartment. By rotating the mirror to the emission monochrometer, the phosphorescence signal emitted from the sample was reflected to the detection system. Since fluorescence lifetimes (10^{-8} - 10^{-6} s) are much shorter than phosphorescence lifetimes (10^{-3} - 10 s), the possible emission of fluorescence from the sample was no longer present at the moment of RTF detection. The scattered light was greatly reduced by inserting the rotating mirror into the Aminco-Keirs phosphorescope. Samples were

spotted drop by drop onto the moving substrate by means of the hypodermic syringe. The substrate consisted of a filter paper strip fed into the sample compartment of the spectrophosphorimeter with the help of a Technicon Continuous Filter AutoAnalyzer. Prior to detection the samples were dried by passing the filter paper strip through a black metal chamber equipped with an infrared lamp. The drying time could be controlled by selecting the paper roll speed with the AutoAnalyzer. The time required for analysis depended upon the optimal drying time which was chosen in accordance to the highest signal-to-background ratio presented for each compound. Most treated phosphors showed the highest ratios when they remained for two minutes in the drying chamber, allowing to perform 7 8 measurements per minute. The RSD of most series of measurements of 10 to 15 identical samples were found to be less than 5%. The LOD were at the nanogram level for pyrene, phenanthrene and chrysene dissolved in a solution of etoAnal-water (1/1, v/v) containing 0.1 M $AgNO_3$, and for PABA dissolved in an aqueous solution of NaOH-NaI (0.5 M-0.25 M). Later, the system was modified by Yan-Sower and Winefordner [73]. The new drying unit consisted of an L-shaped aluminum tube heated with heating tape wrapped with asbestos, and placed directly above the sample compartment of the Aminco-Bowman spectrofluorimeter. A new filter paper guide, which was made from a cylindrical aluminum block, was mounted inside the sample compartment by means of two adjusting screws. A mechanized chopper and an analog switch phosphoroscope were incorporated for time resolution. The frequency of the mechanical chopper for the excitation light was 100 Hz, the gate period of the analog switch was set at 4 ms, and the delay interval between the excitation pulse and the detection was set at 0.2 ms. The analog switch system allowed better control of time resolution than did the rotating mirror. RSD of about 5% and LOD in the nanogram level were obtained for pyrene and phenanthrene in ethanol-0.1 M $AgNO_3$, and for PABA in 1 M NaOH - 1M NaI. Although considerable reduction of analysis time with no loss in sensitivity was also achieved with this system, the samples were still manually spotted with a hypodermic syringe as the

moving filter paper. For each measurement, the filter paper was stopped and the phosphor spot was aligned with the excitation beam. The maximum RTP emission was then obtained by rotating the cylindrical filter paper guide into the sample compartment of the instrument.

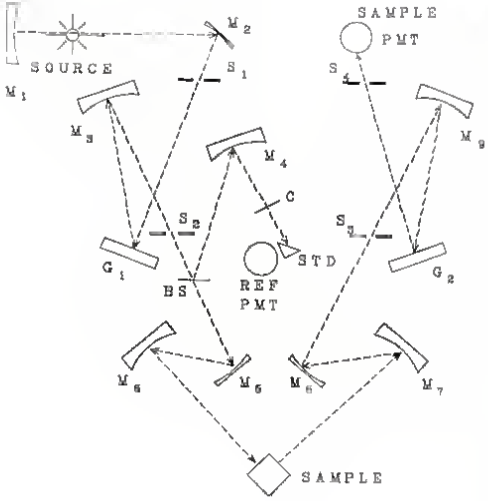
This study concerns the development of a new experimental system for the automation of SSRTP. The manual steps remaining in the previously described systems [45,73] have been eliminated. Sample and heavy atom solutions are continuously sprayed onto moving filter paper strips by means of nebulizers. A new sample holder was designed and adapted to the cell compartment of a Perkin-Elmer LS-5 spectrofluorimeter coupled to a Model 3600 data station. Sixteen to twenty measurements per minute are easily taken, reducing the sampling time usually required for SSRTP analysis. Comparable RSD and LOD to those previously reported [45,73] were obtained, showing that the proposed system is a convenient approach towards the automation of SSRTP.

Experimental

Instrumentation

The RTP spectra and intensity measurements were collected with a Perkin-Elmer LS-5 luminescence spectrofluorimeter (Perkin Elmer, Norwalk, CT) interfaced to a model CLS-3600 data station. A simple diagram of the data-collection unit is depicted in Figure 3-1 [74]. This spectrometer measures the fluorescence or phosphorescence of luminescent samples either in a continuous scan over the selected wavelength range (250-800 nm) of interest or at preselected points within the range. The source is a pulsed xenon flash tube that is of high intensity, short duration, and covers the ultraviolet-visible region. Energy from the source is collimated and reflected onto the entrance slit of the excitation monochromator (1200 lines per mm grating). All wavelengths of excitation energy are diffracted by the grating, and a portion is passed to the beam splitter. At the beam splitter, a small fraction of the energy is directed through a compensator onto the reference cell containing a solution of Rhodamine 101 dye. The compensator compensates for the

Figure 3-1 Schematic diagram of a Perkin Elmer LS 5 Spectrofluorimeter
M mirror
S slit
G grating
BS beam splitter
C comparator
REF PMT reference photomultiplier tube
SAMPLE PMT sample photomultiplier tube
STD standard


SAMPLE
PMT
SOURCE
M 2
S 1
S 4
M 1
M 3
M 4
M 9
C
S 2
S 3
G 1
STD
G 2
BS
REF
PMT
M 5
M 6
M 8
M 7
SAMPLE

varying reflectivity of the beam splitter The rhodamine dye absorbs energy in the 230 630 nm range and fluoresces at about 650 nm with constant quantum efficiency This fluorescence is directed onto the reference photomultiplier detector (PMT) The major portion of the excitation beam is focussed by the mirrors onto the entrance slit of the emission monochromator (960 lines per mm grating) A narrow band of energy is diffracted by the grating through the exit slit and onto the sample PMT The outputs of both the sample and reference paths are processed by ratio recording electronics to produce an output that is proportional to the emission from the sample that has been corrected for variations in the intensity of the source with respect to wavelength The emission intensity and corresponding excitation and emission wavelengths selected by the monochromators are indicated digitally and the corresponding outputs can be read from a printer (digital) and recorder or computer (analogue) Time discrimination was used to distinguish between fluorescence and phosphorescence emission A pulse delay time of 0 03 ms and a gate time for collection of data of 9 0 ms were used for all phosphorescence measurements The excitation and emission slits were set at 5 and 10 nm respectively The original sample holder of the instrument was replaced by a laboratory-constructed filter paper guide (Figure 3 2) Two rails on the base of the filter paper guide allowed the vertical block to slide back and forth which provided a track for the filter paper strip to run smoothly and continuously through the sample compartment of the detection unit The position of the irradiated area on the filter paper strip for maximum RTF signal could be optimized by sliding the vertical block back and forth After obtaining the optimum position, the vertical block could be firmly fastened by means of a set of screws to remain in a constant position during all experiments

Two different ways of spraying solutions on the filter paper strip were tested for best results in the original design which can be seen in Figure 3 3, a conventional atomic absorption nebulizer was employed to spray the analyte solution previously mixed with the heavy-atom enhancer

Figure 3.2 Schematic diagram of a filter paper guide used for a continuous solid substrate room temperature phosphorimetry sampling system (a) Front view of the filter paper guide facing the excitation source of the spectrofluarimeter (b) Back view of the filter paper guide facing the AutoAnalyzer continuous flitter (c) Under view of the filter paper guide

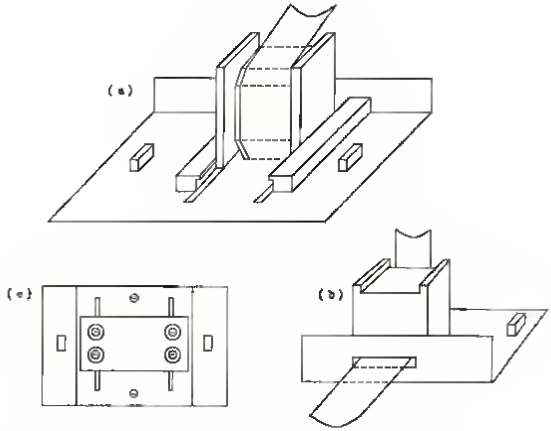
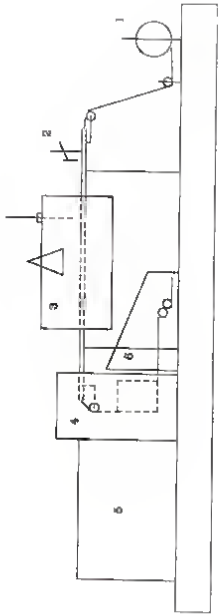

(a)
(c)
(b)

Figure 3-5 Side view of an automatic sampling system for continuous solid substrate room temperature phosphorimetry analysis employing an atomic absorption nebulizer as a solution introduction device (1) Filter paper roll (2) Atomic absorption nebulizer (3) Drying chamber (4) Spectrofluorimeter, (5) Modified sample compartment (6) AutoAnalyzer continuous filter


1
2
3
4
5
6

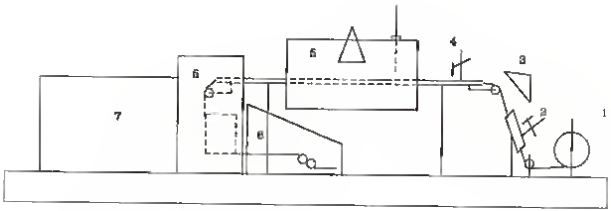
The drying unit consisted of an aluminum chamber equipped with an infrared lamp and a thermometer to monitor the temperature (~75°C). Slits in opposite sides of the drying chamber permitted two parallel metallic rails to pass through forming a track for the filter paper. A groove 5 mm wide and 4 mm deep in each rail held the position of the filter paper constant with respect to the nebulizer. Four metallic posts raised the rails and the drying unit above the level of the spectrofluorimeter. Both rails entered the detection unit, and a Technicon AutoAnalyzer continuous filter (Technicon Instruments Corp., Tarrytown, NY) was used to pull the filter paper through the sample compartment. Metallic rollers located in appropriate positions (see Figure 3-3) helped the filter paper to run smoothly through the system.

The second approach is a modification of the original design and utilized two concentric nebulizers to separately spray the heavy atom and the analyte solutions onto the moving filter paper strip. A schematic diagram of the design can be seen in Figure 3.4. Prior to deposition of analyte solutions on the substrate, the heavy atom solutions were dried onto the filter paper strips by means of an infrared lamp. The relative positions of heavy atom and analyte nebulizers were optimized in such a way that the analyte solutions were sprayed on the same area of filter paper previously sprayed with heavy-atom solutions.

Reagents

PABA (p-aminobenzoic acid) and carbazole (Eastman Kodak Co., Rochester, NY), pyrene (City Chemical Corp., NY), 1,2,5,6-dibenzanthracene (1,2,5,6 DBA), phenanthrene and fluoranthrene (Aldrich Chemical Co., Milwaukee, WI) were reagent grade and used as received. Thallium nitrate ($TlNO_3$) was obtained from Mallinkrodt (St. Louis, MO) and sodium lauryl sulfate (purity of 97%) was purchased from Sigma Chemical Co. (St. Louis, MO). Thallium lauryl sulfate was prepared in our laboratory. See Procedures. Silver nitrate ($AgNO_3$) was purchased from Agenc Chemical Laboratories, Inc. (Redmond, WA). Sodium iodide was obtained from Fisher Scientific Co., (Fair Lawn, NJ). Thallium (I) acetate ($TlOAc$) was from

Figure 3-4 Side view of an automatic sampling system for continuous solid substrate room temperature phosphorimetry analysis employing two concentric nebulizers as solution introduction devices (1) Filter paper roll (2) heavy-stem nebulizer, (3) Infrared lamp, (4) Analyte nebulizer, (5) Drying chamber, (6) Modified sample compartment, (7) Spectrofluorimeter, (8) AutoAnalyzer continuous filter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Aldrich Chemical Co. (Milwaukee, WI) and lead (II) acetate ($PbOAc$) from Allied Chemical (Morristown, NJ). Absolute ethanol (Florida Distillers Co., Lake Alfred, FL) and "nanopure" demineralized water, Barnstead Sybron Corp., (Boston, MA), were used to prepare all solutions. S&S 593-C and 2043-A filter paper (Schleicher and Schuell, Keene, NH) were obtained as 2.5 cm wide and 24 m long filter paper rolls to be used as substrates.

Procedures

Automatic system employing an atomic absorption nebulizer.

Preparation of TlLS. TlLS was prepared by a procedure similar to Humphrey-Baker et al. [22]. Aqueous solutions of 0.25 M sodium lauryl sulfate (NaLS) and 0.25 M $TlNO_3$ were prepared. A 250 mL aliquot of the NaLS solution was mixed with 500 mL of $TlNO_3$ solution. The mixture was stirred and gently warmed for approximately 2 h. Removal from the heat and subsequent cooling of the mixture to room temperature initiated formation of the white precipitate, TlLS. The precipitate was collected after 10 h, isolated by gravity filtration, washed with deionized water and allowed to air dry for 24 h. TlLS was then recrystallized from water until the melting point of TlLS became constant at 197°C. A 60 percent yield of colorless crystalline product was obtained.

Preparation of Solutions. A mixture of ethanol/water 80:20 v/v was used to prepare 0.033 M TlLS and 0.1 M $TlNO_3$ solution. 1 M NaI and 0.1 M $AgNO_3$ solutions were prepared in ethanol/water 50:50 v/v. The heavy atom solutions were then used as solvents for standard solutions of PARA, carbazole, and pyrene.

Measurement Procedure. The initial volume of the sprayed solutions was 1 ml. Flow rates were adjusted to deliver 6 μL of solution per cm^2 of filter paper (20 cm/min substrate speed). After a section of filter paper of the desired length had been sprayed, the solution flow was stopped and the sprayed section was drawn into the drying chamber and remained for approximately 3 min. The RTP signal was then measured. After each measurement, the excitation slit was closed, the filter paper was moved and a new spot of the sprayed section was passed in front of the excitation slit. Since the length of the irradiated area was

approximately 1 cm we were able to take several measurements for each section of filter paper sprayed All the measurements were performed in the presence of dry nitrogen at the maximum excitation and emission wavelengths of each analyte The background signal was obtained by measuring the RTP intensity of the filter paper sprayed with the heavy atom solution at the respective maximum excitation and emission wavelength of each analyte

Automatic System Employing Two Concentric Nebulizers

Preparation of Solutions A mixture of ethanol/water 50 50 v/v was used to prepare 1 M NaI 0 5 M PbOAc and 0 1 M TlOAc solutions, all analyte solutions were prepared in absolute ethanol by serial dilution of 500 µg/mL stock solutions

Measurement Procedures The heavy atom and analyte solutions were simultaneously sprayed onto the moving filter paper strip in both cases the initial volumes were 1 mL, and the flow rates of both nebulizers were adjusted to deliver 5 µL of solution per cm of filter paper strip (20 cm/min filter paper speed) The same measurement procedure as the one previously described for the one nebulizer system was then employed through all experiments

Results and Discussion

The performance of the continuous sampling system employing an atomic absorption nebulizer was evaluated by coating three known phosphorescent compounds carbazole FABA and pyrene Initially 1 M NaI 0 1 M $AgNO_3$ and 0 1 M $TlNO_3$ were tested as heavy atom enhancers However clogging of the nebulizer was observed which affected the reproducibility of the measurements This problem was a result of the high concentrations of the heavy atom salts utilized A dilution of the heavy atom salts was possible but not convenient since the best RTP enhancements with those perturbers are obtained at those higher concentrations [4 59]

In recent studies [71 72] we demonstrated that TlLS is a suitable heavy atom perturber for SSRTP analysis of polynuclear hydrocarbons even

when it is present at low concentrations (0.016-0.065M). As a result 0.033M TiLS was employed as a heavy-atom perturber instead of the former mentioned salts. Table 3.1 lists the analytical figures of merit (AFOM) of the three compounds studied. The calibration curves were linear over 2 decades, and the slopes of the log-log calibration plots were all close to unity, with excellent correlation coefficients. The absolute detection limits were calculated under the assumption that the amount of spray being used was 6 μL of solution per cm of filter paper, which corresponded to the length of the irradiated area in the sample compartment. Possible solution losses were not taken into consideration, the assumption being that the complete volume had been deposited onto the filter paper. LOD in the nanogram range were obtained for PABA and pyrene, and a value approximately 10 times lower was found for carbazole. The precision of the method for all the analytes was less than 7% which was statistically the same as the precision of the background emission. Our results were comparable to those previously reported in the literature and obtained by manual procedures commonly used in SSRTF analysis [4,59,71,72]. However, much shorter analysis times were achieved when the proposed sample holder and the ozonic absorption nebulizer were used to perform the measurements. Calibration curves resulting from six measurements each of six solutions of different analyte concentrations were obtained in less than 3 hs of analysis time. Measurement of the precision for the method which is usually a long and tedious experiment, was accomplished in less than 30 min. By a reduction in drying times, faster analysis could be performed, but no experiments were done in order to optimize this parameter.

An important disadvantage of the proposed system, though, was the clogging of the nebulizer by high concentrations of heavy atom salts. In SSRTF analysis, maximum signal enhancements are usually obtained with perturber concentrations ranging from 0.1-2 M [4,59]. A convenient automatic system, therefore, should present the versatility to work with heavy-atom solutions at their optimum concentration ranges to obtain the best sensitivity for the widest variety of phosphors. As a corrective

Table 3-1 Analytical Figures of Merit for Solid Surface Room Temperature Phosphorimetry With an Automatic Sampling System Employing an Atomic Absorption Nebulizer as Solution Introduction Device

Compound	λ_{ex}(nm)	λ_{em}(nm)[a]	LDR[b]	Slope log log	Correlation Coefficient	LOD[c,d] (μg/ml)	Precision[e] Method(%)
PABA	286	430	1 250	1.00	0 998	0 67(4 02)	3 9
Carbazole	285	416 440 (465)	0 5 100	0 98	0 997	0 079(0 47)	4 7
Fytona	336	523 653	1 250	0 87	0 996	0 35(2 1)	6 [illegible]

[a]Wavelength of maximum RTP emission is underlined when there is more than one component; the wavelengths of the shoulders are in parenthesis the precision for the wavelengths is ±0 5 nm
[b]Linear dynamic range resulting from six measurements of each analyte concentration (μg/mL)
[c]Limit of detection calculated by $3s_{blank}/m_{linear}$
[d]Values in parenthesis are detection limits in ng
[e]Precision for the method was obtained by the following formula

$$P_{method} = \frac{\sqrt{s_{A+B}^2 + s_B^2}}{I_{A+B}} \times 100$$

Sixteen determinations of 10 ppm analyte and their respective blanks were utilized to determine s_{A+B} standard deviation of analyte and blank intensity s_B standard deviation of blank RTP intensity and I_{A+B} average net analyte RTP intensity

means to achieve the required versatility a concentric nebulizer for the deposition of heavy atom solutions was tested. The more homogeneous paths produced by the concentric nebulizer on the filter paper strips encouraged us to utilize a similar one to spray the analyte solutions replacing the atomic absorption nebulizer employed in the original system. An infrared lamp placed between the two nebulizers avoided the results of excessive the analyte solutions on wet substrate, restricting the possible increase of analyte migration to the edges of filter paper strips. The versatility of the modified system was then evaluated by testing three heavy-atom solutions (1M NaI, 0.5 M PbOAc and 0.1M TlOAc) which are commonly used in SSRTP analysis to determine a large variety of compounds at the nanogram level [45,56,58]. Table 3.2 lists the AFOM obtained with these heavy-atom perturbers on six well known phosphorescent compounds including FASA rerbacole pyrene 1,2:5,6-dibenzanthracene (1,2:5,6-DBA), phenanthrene, and fluoranthrene. The LOD were calculated considering that 6 μL of solution were sprayed per cm of filter paper which corresponded to the length of the irradiated area in the sample compartment. Possible volume losses were not taken into consideration assuming that the sprayed volume had been deposited onto the filter paper. Their values were in the nanogram level, which compare favorably with those reported in the literature [4,45,59,73]. With the exception of 1,2:5,6-DBA and fluoranthrene, all the analytes showed calibration curves with LDR over 2 orders of magnitude. In all cases, the slopes of the log-log calibration plots were close to unity with excellent correlation coefficients. The precision of the method ranged from 5.2 to 10% which is within the range usually observed for RTP analysis [4,18,44].

Conclusions

This study showed that the proposed system is a considerable advance towards the automation of SSRTP. The manual steps remaining in previously reported automated systems [45,73] were eliminated. In these systems [45,73], the solutions were spotted onto the moving filter paper by means of a microliter syringe and the optimum position for maximum RTP signal

Table 3 2 Analytical Figures of Merit Observed for Room Temperature Phosphorimetry With an Automatic Sampling System Employing Two Nebulizers as Heavy Atom and Analyte Introduction Devices

Compound	Filter Paper	Heavy Atom	λ_{ex} (nm)	λ_{em} (nm)	LDR[a] (ng)	Slope log log	Correlation Coefficient	LOD[b] (ng)	Method (%)
PABA	593 C	1 M NaI	288	432	5 500	0 96	0 999	1 45	7 4
CARBAZOLE	593 C	1 M NaI	296	440	5 500	1 03	0 999	0 35	8 0
PYRENE	593 C	0 1 M TlAc	343	595	5 400	0 95	0 999	4 00	8 4
1 2 5 6 DBA	2043 C	0 1 M TlAc	300	565	2 5 50	1 00	0 999	0 50	9 6
PHENANTHRENE	2043 C	0 5 M $Pb(Ac)_2$	285	505	5 500	0 96	0 999	4 52	5 2
FLUORANTHENE	2043 C	0 5 M $Pb(Ac)_2$	365	545	25 400	0 98	1 000	4 65	[illegible]

a Linear dynamic range resulting from six measurements of each analyte concentration

b Limit of detection calculated by $3s_{blank}/m$ where s_{blank} is the standard deviation of 16 blanks [illegible] slope of the analytical calibration curve plotted on linear linear coordinates

c Precision for the method was obtained by the following formula

$$\%RSD = \frac{\sqrt{s_{A+B}^2 - s_B^2}}{\bar{I}_{A+B}} \times 100$$

Sixteen determinations of 10 ppm analyte and their respective blanks were utilized to determine s_{A+B} standard deviation of analyte + blank intensity s_B standard deviation of blank RTP intensity and $\bar{I}_{A+B}$ average net analyte RTP intensity

had to be obtained for each sample by aligning the sample spot on the substrate with the excitation beam of the spectrophosphorimeter. Although completion of 8 measurements per minute was reported [45], the optimization step for maximum RTP signal was tedious and required a certain degree of experimenter's expertise.

The new sample holder employed in our system allowed the strip of filter paper to run at a constant position through the sample compartment of the spectrofluorimeter. The optimum position for maximum RTP intensity was easily obtained by sliding the vertical block of the sample holder to coincide with the sprayed path of filter paper and the irradiated area of substrate. The vertical block position and the relative positions of both nebulizers were then kept constant through all experiments, avoiding the need of optimization for each measurement. Sixteen to twenty measurements were easily taken in less than one minute, and six point calibration curves were obtained in approximately two hours of analysis time. Clogging problems with heavy-atom solutions were solved by employing the appropriate nebulizer, providing the automated system with the versatility needed to perform rapid SSRTP analyses. Comparable RSD and LOD to those previously reported by manual [4 44] or automated [45 73] procedures were obtained with shorter analysis times, showing that the proposed system is a convenient approach to develop the full potential of SSRTP as an analytical technique for routine analysis. In addition, the successful performance of the two nebulizer system suggested the possibility of using SSRTP as a detection technique for HPLC. The exit of the chromatographic column can be easily adapted to the analyte nebulizer by means of a connecting tube. The use of a different nebulizer for heavy-atom dependions and the fact that a large portion of these solvents are previously evaporated to deposit the analyte minimizes possible compatibility problems with the mobile phase employed for separation.

CHAPTER 4

SOLID SURFACE ROOM TEMPERATURE PHOSPHORESCENCE DETECTION FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Introduction

In some cases, the selectivity of SSRTF allows the qualitative and quantitative analysis of multicomponent mixtures without previous separation. When the desired compound is the predominant phosphor of the mixture, the excitation and/or emission spectrum of the sample can be directly used for detection purposes [34,61]. Von Wandruszka and Hurtubise [54] employed sodium acetate as a solid substrate to determine the concentration of PABA in vitamin tablets. The tablets investigated also contained vitamins B_1, B_{12}, B_6, and B_{12}, folic acid, pantothenic acid, niacinamide, inositol, choline bitartrate, and biotin. The method was so specific for PABA, that the tablet extract exhibited RTP excitation and emission spectra identical to those of pure PABA samples. Bateh and Winefordner [61] reported a simple SSRTP procedure to analyze theophylline and related compounds such as aminophylline, dyphylline, and oxtriphylline in pharmaceutical preparations with no previous separation. The selectivity was achieved because no compound other than the active ingredient being analyzed presented RTP under the experimental conditions employed.

When the sample contains several compounds with phosphorescence emissions of comparable intensity, the similarity and/or overlapping of broad phosphorescence bands usually interferes in the characterization process of single components. Several alternatives have been proposed to overcome this problem [59,75,76]. Selective external heavy atom perturbation (SEHAP) has been employed for the analysis of target compounds in mixtures without previous separation. Vo-Dinh and Hooyman [59] were able to determine benzo[a]pyrene at the nanogram level in a

synthetic mixture containing acridine, carbazole, dibenzocarbazole and flusranthrene in about 200-fold excess. The high selectivity was the result of using a properly selected heavy atom enhancer (lead (II) acetate) in combination with a convenient set of measurement wavelengths (390, 690nm) which strongly eliminated spectral interference from other components of the mixture. Asafu-Adjaye et al. [75] took advantage of the differences in RTP inducing interactions between phosphors and substrates to achieve better sensitivity with SSRTF. A study of the RTF characteristics of various pesticides showed the possibility of determining naphthaleneacetamide and 3,4-dimethoxybenzaldehyde in the presence of 1,2-diphenylhydrazine, and 1- and 2- naphthols acid without previous separation. When F 81 filter paper, iodide and the excitation/emission maxima wavelengths of naphthaleneacetamide were used in its determination all the other compounds showed very low RTP signal intensities. Similar trends were observed with 3,4-dimethoxybenzaldehyde in mixtures spotted on the same kind of filter paper but in the presence of chloride ion. The single determination of the other insecticides though, was not possible due to interferences from other compounds in the mixtures.

Spectral interference can be considerably reduced by synchronous phosphorimetry. Vo-Dinh and Gammage [76] were the first to introduce the synchronous excitation technique to phosphorescence analysis on solid substrates. A synchronous phosphorescence spectrum is obtained by scanning both the emission (λ) and excitation (λ') wavelengths simultaneously while keeping the spectral interval $\Delta\lambda = \lambda - \lambda'$ constant. If $\Delta\lambda$ is chosen to be equal or larger than the wavelength separation between the lowest singlet and lowest triplet state (Δ_{ST}) of a given compound a synchronous phosphorescence signal of that compound will be observed. On the other hand, the synchronous signals of those compounds obtained with Δ_{ST} values larger than the experimental $\Delta\lambda$ values will not be observed. In addition to the improvement in selectivity provided by Δ_{ST} synchronous phosphorescence spectra have shown narrower bands than

conventional spectra minimizing even more the possible interferences due to spectral overlapping The technique was employed to facilitate the differentiation between dibenzanthracene (DBA) isomers 1,2,3,4-DBA and 1,2,5,6-DBA), which is made difficult by spectral overlapping in conventional SSRTP It was possible to detect 4.2 ng of 1,2,5,6-DBA in a binary mixture containing 10-fold in excess of 1,2,3,4-DBA, showing the potential selectivity of synchronous scanning in SSRTP analysis Its use in routine analysis however strongly depends on optimizing the searching procedure of Δλ values for compounds of interest Future work needs to be done in this area to extend its application to the wide variety of phosphors existent

Although the alternatives described above improved the selectivity of SSRTP in specific situations separation prior to RTP detection is usually convenient when dealing with complex matrices Several workers have proposed paper and thin layer chromatography (TLC) as separation techniques for SSRTP detection [36,77]. Ford and Hurtubise [36] utilized silica gel chromatoplates as solid substrates for the RTP analysis of phthalic isomers DeLima and Nicole [77] employed paper chromatography (PC) to separate synthetic mixtures of 1,8-naphthyridine and some of its derivatives The separations were performed on filter paper strips which were driven into the sample compartment of the spectrofluorimeter by means of a laboratory constructed paper chromatography accessory The unique possibility of complete analysis of complex mixtures using the same substrate turns PC-SSRTP or TLC-SSRTP combinations into relatively simple and useful tools for analytical purposes

SSRTP has also been associated with HPLC [4,78] Filter paper discs were utilized by Vo Dinh and Hiromoto [4] to quantitatively determine benzo[g]pyrene and benzo[e]pyrene in coal liquid samples Ford and Hurtubise [78] used silica chromatoplates to identify benzo[f]quinoline and phenanthridine in shale oil samples In both cases [4,78] the HPLC fractions were manually collected and spotted onto the substrates for SSRTP detection

In this study, the two nebulizer systems described in Chapter 3 for the automation of SSRTF is optimized as a selective detector for HPLC. As a preliminary step for the use of the automated device in a flowing system such as HPLC flow injection analysis (FIA) was employed as a continuous sample introduction approach. Three well known phosphorescent compounds were successfully tested, showing the feasibility of using the automated system in a continuous mode. The performance of the SSRTF system for HPLC detection was then evaluated by comparing the chromatographic parameters and the AFOM of five compounds obtained with the automated system to those obtained by UV absorbance detection. The versatility and selectivity of the SSRTF detector was demonstrated by identifying overlapped compounds either from the SSRTF phosphorogram or from their RTP spectral characteristics.

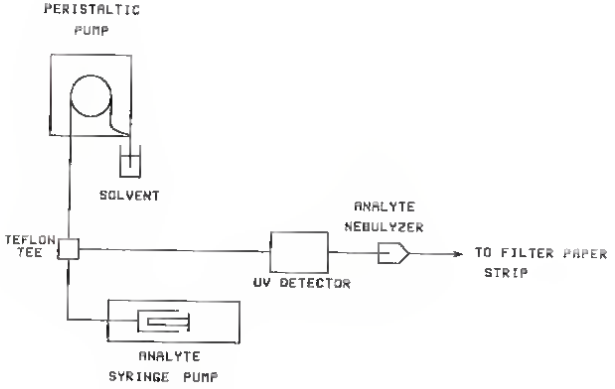
Flow Injection Analysis for Continuous Sample Introduction in SSRTF

Experimental

Instrumentation. Figure 4.1 shows a schematic block diagram of the FIA system used as a continuous sample introduction device. A rabbit peristaltic pump (Rainin Instrument Co., Woburn, MA) and a model 341 syringe pump (Sage Instruments Inc., White Plains, NY) were used to respectively mix the solvent and the analyte solution at different concentration ratios. A Teflon tee (Rainin Instrument Co., Woburn, MA) was employed as mixing chamber and Teflon tubing (0.5 mm i.d.) was used to make all connections. A Beckman model 153 UV detector (254 nm) placed prior to the analyte nebulizer permitted monitoring of the concentration of scintisone sprayed onto the moving filter paper. A pulse delay time of 0.03 ms and a gate time for collection of data of 9.0 ms were used to perform all phosphorescent measurements under a flow of dry nitrogen. The excitation and emission slits were set to 5 nm and 10 nm respectively.

Reagents. All the chemicals were analytical reagent grade and used as received. PABA (p-aminobenzoic acid) was purchased from Eastman Kodak Co. (Rochester, NY) and pyrene, phenanthrene and thallium (I) acetate (TlOAc) were obtained from Aldrich Chemical Co., (Milwaukee, WI).

Figure 4-1 FIA system used as a continuous sample introduction device for automated SSRTF analysis


PERISTALTIC
PUMP
SOLVENT
ANALYTE
NEBULYZER
TEFLON
TEE
TO FILTER PAPER
STRIP
UV DETECTOR
ANALYTE
SYRINGE PUMP

Absolute ethanol (Florida Distillers Co , Lake Alfred FL) and "nanopure" deionized water Barnsted Sybron Corp (Boston, MA) were used as solvents to prepare the solutions S & S 2043 A filter paper (Schleicher and Schuell Trenton NH) was obtained as a 2 5 cm wide x 24 m long filter paper roll and utilized as a substrate

Procedure A 0 1 M TlOAc solution in ethanol/water 50 50 v/v was employed as a heavy-atom enhancer and continuously sprayed onto the moving filter paper at a flow rate adjusted to deliver approximately 10 μL of solution per cm^2 of substrate (20 cm/min filter paper speed)

By keeping the flow rate of ethanol (0 0966 mL/min) constant and varying the flow rate of analyte solutions it was possible to obtain a set of concentrations ranging from 2 5 μg/mL to 141 μg/mL Since we were interested in maintaining an approximately constant volume of solution

sprayed on the substrate (10 μL/cm^2) 100 μg/mL and 500 μg/mL stock analyte solutions were employed for the lower (2 5-28 25 μg/mL) and upper part of the calibration curves respectively Table 4-1 lists the different syringe pump flow rates used with the FIA system the total flow rate the analyte concentration of the sprayed solutions and the mass of analyte deposited per cm^2 of filter paper

All the measurements were performed in a continuous mode with the use of a kinetic program which permitted plotting the phosphorescent intensity as a function of sampling time A total sampling time of 80 s and a data interval of 0 2 s were employed to obtain 400 points per "FIAgram" While the desired section of sprayed filter paper was passing through the sample compartment of the spectrofluorimeter the excitation slit was opened and the RTF emission at the maximum excitation and emission wavelengths of the researched analyte was registered Considering the filter paper speed 80 s of sampling time allowed the registering the phosphorescent intensity of 28 cm of sprayed substrate Since the length of irradiated area was approximately 1 cm the equivalent of 28 different samples were measured during that period of time The

Table 4 1 Analyte Concentrations Sprayed Onto the Moving Filter Paper With a Flow Injection Analysis System as a Continuous Sampling Introduction Device for Solid Substrate Room Temperature Phosphorimetry

FRSP[a] (mL/min)	TFR[b] (mL/min)	c_s[c] (μg/mL)	Mass of Analyte[d] (μg/cm²)
$2\ 4 \times 10^{-3}$	$9\ 90 \times 10^{-2}$	2 5	0 023
$5\ 0 \times 10^{-3}$	$1\ 02 \times 10^{-1}$	5 0	0 048
$1\ 34 \times 10^{-2}$	$1\ 10 \times 10^{-1}$	12 4	0 128
$3\ 74 \times 10^{-2}$	$1\ 34 \times 10^{-1}$	28 2	0 355
$9\ 6 \times 10^{-3}$[e]	$1\ 06 \times 10^{-1}$	46 0	0 460
$2\ 00 \times 10^{-2}$[e]	$1\ 17 \times 10^{-1}$	87 0	0 857
$3\ 74 \times 10^{-2}$[e]	$1\ 34 \times 10^{-1}$	141 0	1 777

[a]FRSP = flow rate of stock analyte solutions from the syringe pump
[b]TFR = total flow rate of analyte solutions Calculated by adding the solvent flow rate to FRSP
[c]Concentration of analyte solutions sprayed on the substrate
[d]Mass of analyte deposited per cm² of filter paper Calculated by the following equation (TFR/A) x c_s, where A = 10 5 cm²/min is the area of substrate sprayed per min taken into consideration the speed of the moving paper (21 cm/min) and the width of the sprayed path (0 5 cm)
[e]Stock solution concentration 500 μL/mL instead of 100 μL/mL used for the first four flow rates

of the filter paper sprayed with heavy-atom and ethanol solutions at the maximum excitation and emission wavelengths of each analyte. No significant difference in the background signal was observed for the different flow rates used for calibration curves.

Results and Discussion

The performance of the continuous sampling system was evaluated by tracing three well-known phosphors which have been extensively examined by SSRTP [4]. The choice of PABA phenanthrene and pyrene as probe compounds allowed comparison between the performance of the system operated in a continuous mode and that obtained with the procedure described in Chapter 3, when the filter paper strip was stopped for signal detection. 0.1 M TlOAc was selected as a heavy atom perturber, being particularly efficient in the enhancement of the phosphorescent emissions of polyaromatic hydrocarbons (PAHs) such as pyrene and phenanthrene [44].

Table 4-2 lists the AFOM obtained with the FIA system as a continuous sample introduction device. Each phosphorescent intensity plotted in the calibration curves is the average of 12 replicates taken from the FIAgrams at every 5 s between 60 s and 120 s of sampling time. The amounts of analyte responsible for the phosphorescent signal were calculated from the values listed in Table 4-1 ($\mu g/cm^2$) considering an irradiated area of 0.5 cm^2. The limits of detection were obtained from the equation $c_L = k s_b / m$ where s_b is the standard deviation of the blank during the sampling time, k=3 and m is the slope of the analytical calibration curve plotted on linear-linear coordinates [49].

The precision of the method was determined from the formula $P = (s_{A+B} + s_B)^{\frac{1}{2}} / I_{A+B} \times 100$ where s_{A+B} is the standard deviation of 12 measurements of the phosphorescent intensity of 128 ng of analyte+blank, s_B is the standard deviation of the blank and I_{A+B} is the average net analyte signal.

With the exception of pyrene, the other analytes resulted in calibration curves with linear dynamic ranges of approximately 2 orders of magnitude. In all cases, the slopes of the log-log calibration plots were close to unity with excellent correlation coefficients. The LOD were in

Table 4-2 Analytical Figures of Merit Obtained with Flow Injection Analysis System as a Continuous Sampling Introduction Device for Solid Substrate Room Temperature Phosphorimetry Analysis

Compound	Ex (nm)	Em (nm)	LDR[a] (ng)	Slope log-log	Correlation Coefficient	LOD[b] (ng)	Precision of Method (%)
PABA	288	432	11 5 - 888 5	1 05	0 996	3.71	12 78
Phenanthrene	284	505	24 0 - 888 5	0 95	0 999	2.34	12 65
Pyrene	343	595	24 0 - 230 0	1 05	0 993	4.98	10 62

[a]LDR = Linear Dynamic Range
[b]LOD = Limit of Detection

the nanogram range which compares favorably with the ones reported in the literature [4] and with those obtained by stopping the filter paper at the measurement time (see Chapter 3) The precision of the method however was a little poorer than expected The use of FIA as a solution-handling technique offered no environment for a highly reproducible mixing of components where microvolumes were conveniently manipulated and metered [79]. The RSD obtained though was higher than those obtained in Chapter 3 when a batch procedure was employed to prepare the solutions sprayed onto the substrate (5 2 - 8 4%) Several reasons can be given for the higher variations observed Since in the actual procedure the measurements were taken in a continuous mode the noise caused by the motion of the paper and possible concentration fluctuations induced by the peristaltic flow could explain, to some extent, the observed results Due to limitations of the peristaltic pump employed smaller volumes than 10 µL per cm of filter paper could not be sprayed This is approximately twice the volume utilized in the procedure described in Chapter 3 The deposition of large volumes (>6 µL) on filter paper can cause the migration of the analyte to the edges of the substrate [4,69 70 73] Since the migration is not uniform and the irradiated area on the substrate was kept constant through all measurements the spreading of analyte on a larger area of substrate could be responsible also for the higher variations observed in RTP signals Finally, the larger volume of solvent sprayed on the filter paper and the fact that the drying time remained the same as the one used in Chapter 3, could lead to greater quenching by moisture of analyte signals deteriorating the precision of the method

The higher RSD observed indicates the possibility that the precision of the method is mainly related to phenomena occurring at the surface of the substrate instead of imprecisions existent in batch procedures The use of a different peristaltic pump which would allow the deposition of convenient volumes of solution (≤ 6 µL) on the filter paper strip would probably be more suitable for FIA-SSRTP analysis However the FIA-SSRTP

system employed in a continuous mode produced comparable LOD and LDR to the ones obtained in Chapter 3 showing the feasibility of using the SSRTF automated system as a selective detector for HPLC analysis

Utilization of the Two Nebulizer Automatic System as a Continuous SSRTF Detector for HPLC AnalysisExperimental

Instrumentation The HPLC system consisted of an Altex model 110A pump used with a Rheodyne valve and a 20 µL sample loop The Altex column was 15 cm long, 4 6 mm I d , and filled with ultrasphere ODS (5µm particle diameter) The UV detector was an Altex model 153 with a cell volume of 8 µL response time constant of 1 s and fixed wavelength at 254 nm The two nebulizer system for SSRTF detection was employed without previous modifications The eluent from the chromatographic column was sprayed onto the moving filter paper strip by connecting, with a Teflon tube (0 25 mm I d), the exit of the UV detector cell to the analyte nebulizer of the automatic system The Teflon tube was kept as short as possible (<15 cm) to minimize band broadening All the SSRTF measurements were performed under a flow of dry nitrogen with a delay time of 0 03 ms and a gate time for collection of data of 9 0 ms The excitation and the emission slits were set to 10 nm and 5 nm respectively

Reagents All chemicals were analytical reagent grade and were used as received Carbazole was purchased from Eastman Kodak Co , pyrene benzo[a]pyrene fluoranthene phenanthrene and thallium (I) acetate (TlOAc) were obtained from Aldrich Chemical Co HPLC grade methanol (Fischer Scientific Co) and "nanopure" demineralized water (Barnsted Sybron Corp) were used throughout S & S 2043 A filter paper (Schleicher and Schuell) was obtained as a 2 5 cm wide x 24 m long filter paper roll and was utilized as a substrate Methanol/water solution of different volume ratios were employed as solvents to prepare 200 µg/mL stock solutions of carbazole and phenanthrene (80 20 v/v), and pyrene and fluoranthene (70 30 v/v) The same mass of benzo[a]pyrene was dissolved in pure methanol A 0 1 M solution of TlOAc in methanol/water 50 50 v/v was employed as a heavy-atom enhancer

Procedure All the RTP measurements were performed in a continuous mode with the use of a kinetic program which permitted plotting the phosphorescent intensity as a function of time While the desired section of filter paper was passing through the sample compartment of the spectrofluorimeter, the excitation slit was opened and the RTP emission at the selected excitation and emission wavelengths was registered The total sampling time was chosen according to the separation time in the chromatographic column and a data interval of 0.2 s was kept constant through all the experiments For all the AFOM, a sampling time of 800 s was employed to obtain 4000 points per chromatogram The mobile phase drying process introduced a delay time of 330 s This delay time in the SSRTF chromatogram corresponded to the time spent by the analyte on the substrate to "travel" from the nebulizer to the excitation slit of the spectrofluorimeter Since the time spent for the effluent to go from the UV detector to the analyte nebulizer was negligible for all the flow rates tested, the same delay time was observed for all the experiments The retention times in the SSRTF chromatograms were then corrected with respect to the observed values (330 s)

Results and Discussion

Five well known phosphorescent compounds carbazole phenanthrene, pyrene, benzo[a]pyrene (B(a)P), and fluoranthene were selected to compare the UV and the SSRTF system for HPLC detection All of these strongly absorbed at 254 nm at which the UV measurements were performed The 0.1 M TlOAc solution chosen as a heavy atom perturber was shown to be particularly efficient in the signal enhancement of most of the probe analytes [4 46]

Figure 4.2 shows a SSRTF chromatogram along with the corresponding classical UV chromatogram of a mixture containing carbazole phenanthrene, pyrene, and B(a)P In order to simplify the measurement procedure only two sets of detection wavelengths were employed The measurement wavelengths selected took into consideration the relative phosphorescent intensity of the studied compounds When necessary, the chosen wavelength

Figure 4.2 UV absorption (a) and solid substrate room temperature phosphorimetry (b) chromatogram showing the separation of a mixture containing 200 pg of phenanthrene and 500 pg of carbazole pyrene and benzo[e]pyrene Mobile phase methanol flow rate 0.44 ml/min sample volume 20 µl

(a)
Carbazole
Phenanthrene
Pyrene
Benzo[a]pyrene
0
2
4
6
8
10
12
14
Time (min)
(b)
Carbazole
Phenanthrene
Pyrene
Benz o[a]Pyrene
0
2
4
6
8
10
Time (min)

pair was set closer to the wavelengths for maximum RTF signal of the weaker phosphor. The wavelength and chromatographic parameters are listed in Table 4.3.

Chromatographic Band Broadening. The peak efficiencies were obtained assuming Gaussian peaks and are listed in Table 4-3. The low efficiency obtained for carbazole (N_{UV} = 400, N_{RTF} = 370) was not surprising. Carbazole, whose other name is dibenzopyrrole is an amine containing basic compound. The NH amine group of the pyrrole ring has a high affinity for surface silanols of the silica stationary phase. This affinity induces a peak tailing and low efficiency. The relatively low efficiency assured for phenanthrene (N_{UV} = 1400, N_{RTF} = 1300) may be due to extra column band broadening effects apart from the detectors. We point out that the chromatographic system was not optimized for maximum efficiency. However it is interesting to estimate the band broadening induced by the SSRTF detector. A 15% efficiency loss was observed in comparing the UV absorption to the SSRTF values (Table 4.3). The band broadening produced by the connecting tubing, nebulizer and moving paper strip can be roughly quantified through variance estimation using the equation $s^2 = V^2_r/N$. Defining σ_{sscrtp} and s_{uv} from SSRTF and the UV absorption chromatograms, the difference $\sigma^2_{sscrtp} - s^2_{uv}$ corresponds to the band broadening due to the SSRTF detector because variances are additive. The estimated value was found to be approximately 900 μL^2 ± 300 μL^2 which corresponded to a dead volume of 30 μL ± 17 μL for the SSRTF detector. It is important to note that, although the moving filter paper strip induced a 330 s response delay for drying and reaching the observation window, it did not produce excessive band broadening. Since the mobile phase and the solvent of the heavy atom solution were evaporated by infrared lamps, the solutes were trapped into the cellulose fibers of the paper which restrained their mobilities. By reducing the UV-SSRTF connecting tubing (16 μL) and using a nitrogen nebulizer it should be possible to minimize the band broadening even more.

Table 4 3 Wavelength Settings Employed and Chromatographic Parameters Obtained by High Performance Liquid Chromatography with UV Absorption and Solid Substrate Room Temperature Phosphorimetry Detection

Solute	λ_{ex} [a] nm	λ_{em} [a] nm	V_r [b] mL	k [c]	Efficiency [d] UV plates	RTP plates
Carbazole	290(296)	470(440)	1 95	1 00	400	370
Phenanthrene	290(285)	470(505)	2 66	1 73	1400	1300
Pyrene	343(343)	585(595)	3 10	2 18	3000	2400
Benzo[a]pyrene	343(335)	585(543)	4 34	3 45	3200	2800
Fluoranthene	343(365)	585(545)	2 92	2 00	3000	2400

[a] The values in parenthesis correspond to the wavelengths for maximum emission The others are the selected wavelengths for the SSRTP chromatograms

[b] Retention volumes (V_r) obtained with a flow rate of 0 443 mL/min

[c] Capacity factors (k) calculated for a dead volume (V_0) of 0 87 mL

[d] Efficiencies calculated using $N=4(V_r/W_{0\,4H})^2$ in which $W_{0\,4H}$ is the peak width at 60% of the peak height expressed in volume units Accuracy 20%

Flow Rate Effect. When a constant ratio between the HPLC flow rate and the strip chart recorder speed was maintained, we observed little variations of UV peak areas with the flow rate for fixed sample weight. Important variations of SSRTF peak areas were observed with flow rate changes. The phosphorescent intensity was very dependant on the flow rate of the mobile phase. Figure 4-3 shows the chromatographic SSRTF responses (SSRTF peak area) versus flow rate. It can be seen that there is an optimum flow rate for maximum RTF signal and therefore for highest sensitivity. Several parameters related to the flow rate variation could be responsible for the observed phenomenon. By keeping constant the filter paper speed, the amount of analyte deposited per surface area of substrate increased with the flow rate. If a peak has a base width of one minute, the corresponding paper length on which the analyte would be sprayed is 21 cm if the peak has a base width of 10 s the same amount of analyte would be sprayed on 3.5 cm and so on. Since the analyte was deposited on a shorter length of substrate it would be reasonable to expect higher phosphorescent signals at high flow rates. However by increasing the flow rate of the mobile phase a larger volume of solvent per substrate length was also delivered. Since the filter paper speed was kept constant the drying time remained the same and could be insufficient for complete drying of the solvent. If this was the case moisture could be responsible, to some extent for quenching the phosphorescent signal. In addition, the increase in the flow rate was followed by an increase in the width of the sprayed area. Since the excitation slit of the spectrofluorimeter was kept constant a possible diffusion of the analyte to the edges of the filter paper could also be responsible for the decrease observed in the phosphorescence intensities. With the exception of pyrene all the other compounds showed the strongest phosphorescent signals between 0.354 mL/min and 0.443 mL/min. Considering the advantage of short analysis time 0.443 mL/min was chosen as the optimum mobile phase flow rate resulting in a total analysis time of approximately 17 min per SSRTF chromatogram.

Analytical Figures of Merit, AFOM. Table 4-4 compares the AFOM obtained with the UV absorption and the SSRTF system for HPLC detection It is important to mention that both detectors were compared under experimental conditions for maximum SSRTF sensitivity and reproducibility The HPLC system was not optimized for UV absorption detection The limits of detection and the reproducibilities of measurements obtained with the UV detector however were comparable with those commonly observed when these kinds of devices are used for HPLC analysis When measured by SSRTF, carbazole and phenanthrene showed calibration curves with larger linear dynamic ranges Pyrene absorbed strongly in the UV and as had a low limit of detection with the UV detector, the SSRTF calibration curve for pyrene had a shorter linear dynamic range in all cases slopes close to unity and satisfactory correlation coefficients were obtained with both systems The limits of detection with the UV detector were calculated from the equation $r_L = 3/5(I_{p-p}/m)$ where I_{p-p} is the peak to peak background noise and m is the slope of the calibration curve [49] The SSRTF values were corrected by multiplying the equation above by the factor A_t/A_s where A_t is the irradiated area in the sample compartment and A_s is the total area of substrate on which the analyte had been sprayed With both detection systems carbazole phenanthrene and B(e)P showed comparable limits of detection (LOD) in the nanogram range The higher LOD value obtained for pyrene with the SSRTF detector can be attributed to the weak phosphorescence signal presented for this phosphor under the experimental conditions employed in the study The relative standard deviations obtained with the SSRTF detector were within the reproducibility range of the technique (4% 15%) and as expected were poorer than those obtained by UV absorption detection (2%-7.5%)

Special Features. Although conventional UV absorption detectors operated at fixed wavelengths are able to discriminate between absorbing and non-absorbing species they lack versatility and selectivity for the determination of absorbing compounds The separation process in the chromatographic column has to be either complete or performed in such a

Figure 4-3 Room temperature phosphorimetry peak area (I_p) variations of carbazole Δ, phenanthrene ○, pyrene •, and benzo[e]pyrene ★ with mobile phase flow rate. Mobile phase: methanol. Sample size: 200 pg of phenathrene, 500 pg of others, injected volume 20 μL.

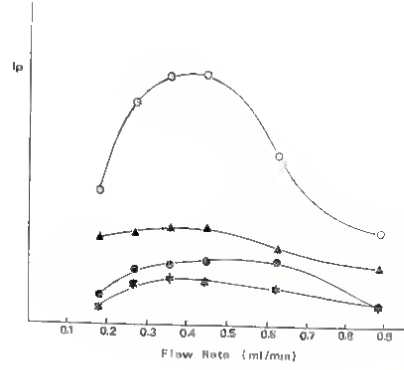

Ip
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Flow Rate (ml/min)

Table 4.4 Analytical Figures of Merit Obtained for High Performance Liquid Chromatography Using Solid Substrate Room Temperature Phosphorimetry and UV Absorption Detection

Solute	LDR[a]	SLOPE[b]	CORRELATION COEFFICIENT	LOD[c] (ng)
Carbazole	3.0×10^3 (6.5×10^5)	1.06 (0.93)	0.983 (0.926)	0.77 (1.26)
Phenanthrene	3×10^3 (2.5×10^5)	1.04 (0.86)	0.999 (0.999)	0.96 (0.98)
Pyrene	2.2×10^2 (2.6×10^3)	0.95 (0.99)	0.985 (0.998)	5.24 (0.46)
Benzo[e]pyrene	3.6×10^2 (3.4×10^5)	1.00 (0.98)	0.983 (0.998)	3.16 (4.62)

The values listed in parenthesis correspond to the AFOM obtained with the UV detector

[a] Linear dynamic range (LDR) was estimated by dividing the upper linear concentration by the limit of detection

[b] Calculated from the curve log phosphorescence intensity (absorbance) versus log concentration

[c] Signal/noise ratio = 3 and volumes of 20 µL (RTP) and 8 µL (UV) were used to estimate the limits of detection (LOD)

way that compounds with minimum overlapping reach the detector. Most times, this is a tedious procedure which usually involves either the optimization of a volume ratio between two solvents or the search for a convenient mobile phase. In addition, the detector wavelength does not usually coincide with the maximum absorbance peak of the desired compound which restricts the sensitivity of HPLC.

Commercially available UV-visible absorption detectors that consist of a scanning spectrometer or a photodetector array with grating optics offer the possibility to select the best wavelength for each analyte. If overlapped compounds reach the detector, the selection of an appropriate detection wavelength for a single component is often difficult due to the normally broad absorbance bands.

Even though broad molecular phosphorescence bands commonly overlap, it is unusual for different compounds to have coincident spectra in such a way that neither excitation nor emission wavelengths can be selected to measure a single component of the mixture. This unique feature of SSRTF is responsible for the high selectivity of the technique [4,59-75-76]. Figures 9 to 11 show the UV chromatogram of a mixture containing fluoranthene and pyrene along with the respective SSRTF chromatograms. The chromatographic parameters of fluoranthene are listed in Table 4-3. From the UV chromatogram (Figure 4.4) it can be seen that complete resolution was not achieved by using pure methanol as the mobile phase. Although the UV absorption detection of either component is possible at these experimental conditions, the example is useful to illustrate the selectivity of the SSRTF detector. By exciting at 343 nm and registering the phosphorescent emission at 535 nm, the SSRTF chromatogram showed the presence of both solutes (Figure 4.5). When the detection wavelengths were shifted to the maximum excitation and emission of fluoranthene, the phosphorescence of pyrene was no longer registered (Figure 4.6) and the single detection of fluoranthene was possible without the presence of pyrene in the chromatogram. Further identification of fluoranthene was then performed by stopping the filter paper strip at the maximum

Figure 4.4 UV absorption chromatogram of a mixture containing 200 pg of fluoranthrene and pyrene. Mobile phase: methanol; flow rate: 0.443 mL/min; sample volume: 20 µL.

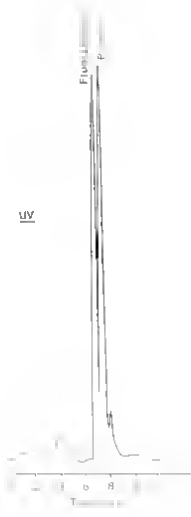

UV

Figure 4 6 Solid substrate room temperature phosphorimetry chromatogram of a mixture containing 200 pg of fluoranthrene and pyrene Obtained under identical experimental conditions of Figure 4-4. Measurement wavelengths 365/585 nm

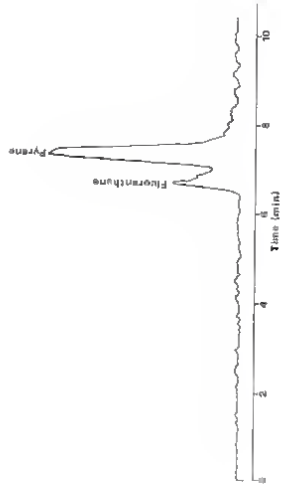
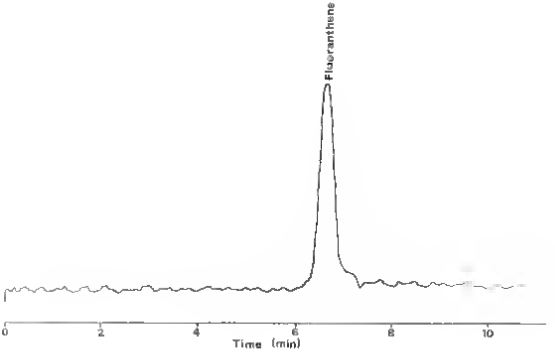

Fluoranthene
Pyrene
Time (min)
0
2
4
6
8
10

Figure 4-5 Solid substrate room temperature phosphorimetry chromatogram of a mixture containing 200 pg of fluoranthrene and pyrene. Obtained under identical experimental conditions of Figure 4-4. Measurement wavelengths 343/585 nm


Fluoranthene
0
2
4
6
8
10
Time (min)

phosphorescent intensity of the first eluted compound and comparing its emission spectrum with the corresponding spectrum of fluoranthene (see Figure 4-7). The spectral identification of pyrene was done in a similar way. The substrate was stopped at the maximum phosphorescent intensity of the second eluted compound and the emission spectrum was registered at the maximum excitation wavelength of pyrene. A spectrum with the same characteristics of the emission spectrum of pure pyrene was obtained (Figure 4-8). Spectral identification was also possible when both compounds were present at the same level of concentration on the substrate. By stopping the filter paper strip at the maximum phosphorescence intensity detected between the elution of the compounds, and irradiating the substrate at the maximum excitation wavelength of pyrene, the presence of both analytes was confirmed by registering two phosphorescence bands with emission maxima at 545 nm (fluoranthene) and 592 nm (pyrene) (see Figure 4-9). When the substrate was irradiated at the maximum excitation wavelength of fluoranthene, the phosphorescence emission of pyrene was no longer detected (Figure 4-10). By comparing Figures 13 and 14, it can be noticed that even a weak phosphor like pyrene could be spectrally identified in the presence of similar concentrations of a strong phosphor like fluoranthene.

Conclusions

In this study, the feasibility of using SSRTP as a detection technique for HPLC has been demonstrated. By using the two nebulizer automatic system, it was possible to perform HPLC analysis in a continuous mode. The proposed detector has been evaluated by comparing its performance to a classical UV absorption detector under the same experimental conditions. A 15% efficiency loss was observed in the SSRTP chromatograms mainly due to the UV absorption SSRTP detector connecting tubing (16 µL), nebulizer and moving filter paper strip. By reducing the length of the connecting tubing and using a micro-nebulizer, it should be possible to minimize the observed band broadening. Comparable AFOM were obtained for both detection modes. By selecting the appropriate

Figure 4-7 (a) Room temperature phosphorescence emission (I_e) spectrum of the first eluted compound using the maximum excitation wavelength of fluoranthrene (365 nm) (b) Room temperature phosphorescence emission spectrum of fluoranthrene obtained under the same experimental conditions of (a)

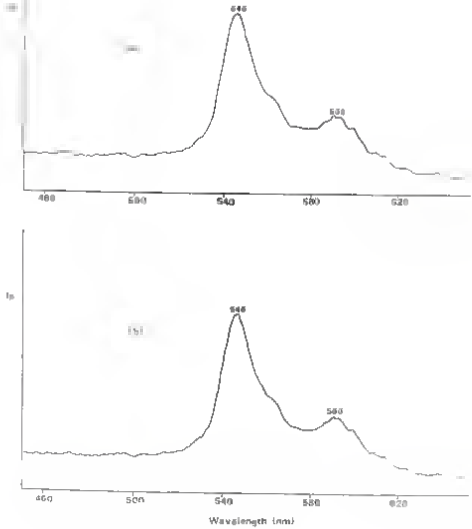

546
580
480
500
540
560
620
546
580
460
500
540
580
620
Wavelength (nm)

Figure 4-8 (a) Room temperature phosphorescence emission (I_p) of the second eluted compound registered at the maximum excitation wavelength of pyrene (343 nm) (b) Room temperature phosphorescence emission spectrum of pyrene obtained under the same experimental conditions of (a)

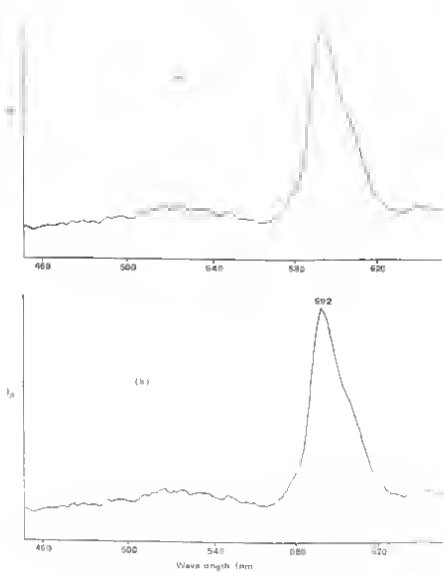

592
(b)
I_A
460
500
540
580
620
Wavelength /nm

Figure 4 9 Room temperature phosphorescence emission (I_p) spectra of the substrate registered at the minimum phosphorescence intensity observed between the elution of the two compounds Excitation wavelength 343 nm

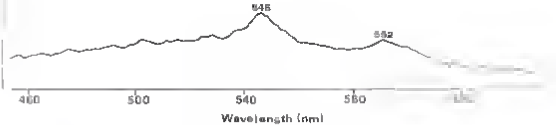
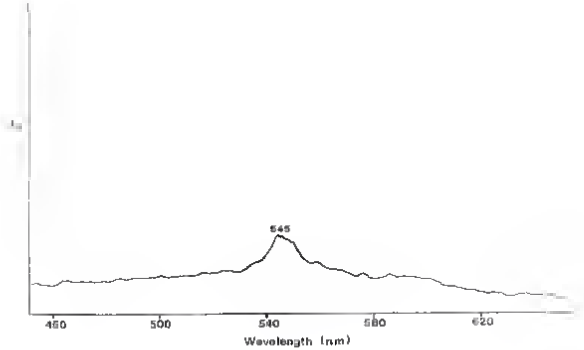

545
552
460
500
540
580
Wavelength (nm)

Figure 4 10 Room temperature phosphorescence emission (I_p) spectra of the substrate registered at the minimum phosphorescence intensity observed between the elution of the two compounds
Excitation wavelength 365 nm


545
460
500
540
580
620
Wavelength (nm)

excitation and emission wavelengths of measurement, it was possible to characterize a single component of a mixture partially resolved by HPLC. This special feature makes the SSRTP detector a useful tool for liquid chromatographic separations. Complex systems can be separated into simpler ones and the individual components of a mixture can be selectively determined by choosing the appropriate set of measurement wavelengths. The versatility of the new detector permitted identification of compounds either from the retention times or from their RTP spectral characteristics, showing its potentiality for qualitative purposes. The greatest versatility, however, would result with the use of an intensified photodiode array detector. This instrument would allow phosphorescence spectra to be recorded as the peaks elute permitting "impure" probes to be resolved into individual components. We also believe that the SSRTP detector could be very useful in areas such as forensic science where a permanent record of the analyzed samples would be extremely helpful. Compounds adsorbed on solid substrates and stored under a nitrogen atmosphere for several weeks have presented constant phosphorescence intensity [20 21]. This offers the possibility of identifying the constituents of a stored sample at any required time. By knowing the position of every compound on the filter paper strip a new chromatogram (phosphorescence versus distance) can be obtained. If further identification is necessary, the substrate can then be stopped at the maximum phosphorescence intensity of every compound to run its excitation and emission spectra. Additional work, however, is necessary in the future to optimize the storing conditions for compounds of forensic interest.

CHAPTER 5

SOLID SURFACE ROOM TEMPERATURE PHOSPHORESCENCE DETECTION OF CAFFEINE, THEOPHYLLINE AND THEOBROMINE IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Introduction

Stimulated by widespread cases of drug abuse by olympic athletes to improve their physical performance and/or mental condition, the International Olympic Committee (IOC) established a Medical Commission (MC) and charged it with the responsibility of controlling the abuse of performance-enhancing drugs [50 51]. The MC developed a list of banned substances which include more than 200 different drugs and metabolites such as psychomotor stimulants, sympathomimetic amines, central nervous system stimulants, narcotic analgesics and anabolic steroids [52]. Almost all banned drugs and metabolites are excreted in urine resulting in screening analysis of a large number of urine specimens. Depending on the type of compounds, gas chromatography (GC), gas chromatography mass spectrometry (GC MS), high performance liquid chromatography (HPLC) and radioimmunoassay methods are usually employed for initial screening of urine samples [52]. In all cases, the finding of a drug or metabolite by a screening procedure must be confirmed by a different analytical method of measurement.

Caffeine (1 3 7 trimethylxanthine) is a nervous system stimulant classified as a banned substance by the MC. Since it is considered to be part of the habitual diet in man, a concentration limit of 12 $\mu g/mL$ is allowed. Concentrations in urine above this limit are considered illicit. The abuse of caffeine by an athlete is usually indicated by GC screening tests. Its confirmation is then performed by HPLC UV absorption detection.

In this study, we are proposing the use of SPLC-SSRTP as an alternative method for the analysis of caffeine and its two

dimethylxanthine (DMX) metabolites, theophylline (1,3 DMX) and theobromine (3,7-DMX) [83,84]. The AFOM obtained with the SSRTP detector are compared to those obtained by UV absorption detection under the same experimental conditions. The SSRTP and UV chromatograms of spiked urine samples are compared, and the feasibility of keeping the SSRTP chromatograms as a permanent record of the analyzed samples is discussed.

Experimental

Instrumentation

The instrumental configuration employed in this study has been described in detail in Chapter 4. All the SSRTP measurements were performed under a flow of dry nitrogen with a delay time of 0.03 ms and a gate time for collection of data of 9.0 ms. The excitation and emission slits were set to 10 nm and 5 nm respectively. When needed a Perkin Elmer phosphorescence standard (300 nm/615 nm) was employed for verification of possible intensity variations of the excitation source. The excitation and emission spectra were scanned at 120 nm/min rate and controlled by a Perkin-Elmer PECLS application program.

Reagents

"Nanopure" demineralized water (Barnsted Sybron Corp.) and analytical reagent grade chemicals were used throughout. HPLC grade methanol was from Fisher Scientific Co. Caffeine, theophylline and theobromine were purchased from Sigma Chemical Co. and used without further purification. Potassium iodide (KI) was obtained from Fisher Scientific Co., thallium (I) acetate (TlOAc) was from Aldrich Chemical Co. and lead (II) acetate (PbOAc) from Allied Chemical. Absolute ethanol (Pharmco Distillers Co.) was used to prepare the heavy atom solutions. Anhydrous $CaSO_4$ (Hammond Drierite Co.) was employed as a drying substance to avoid the presence of humidity in a desiccator. S & S 2043 A filter paper (Schleicher and Schuell) was obtained as a 2.5 cm wide x 24 m long filter paper roll and employed as a substrate. An Acrodisc disposable filter (Gelman Sciences) using 0.45 μm pore size membranes was utilized to filter urine samples.

Procedure

The analyte solutions employed in this study were prepared in nanopure water by serial dilution of stock solutions containing 300 μg/mL of active compound and 3% v/v methanol to aid solubilization. A mixture of ethanol/water 50:50 v/v was used to prepare 1 M KI, 0.5 M $Pb(OAc)_2$ and 0.1 M TlOAc solutions. Fresh urine samples, obtained from volunteers not taking any medications were spiked with known amounts of the drugs. Before injection into the separation column, the spiked urine samples were filtered through an Acrodisc disposable filter assembly. After approximately 50 urine sample injections, partial clogging of the chromatographic column was observed as manifested by changes in retention times of the drugs. The heavy atom solutions were continuously sprayed onto the moving filter paper at a flow rate adjusted to deliver approximately 6 μL of solution per cm^2 of substrate.

All the RTP measurements were performed by a procedure previously described in Chapter 4. A sampling time of 800 s and a delay interval of 0.2 s were employed to obtain 4000 points per SSRTP chromatogram. All SSRTP retention times were corrected with respect to delay times introduced by filter paper speeds.

Results and Discussion

As a tentative means of obtaining a suitable heavy atom enhancer for the SSRTP analysis of caffeine, theophylline and theobromine, a study of their RTP characteristics was performed in the presence of three common heavy atom salts, which included 1 M KI, 0.1 M TlOAc, and 0.1 M $Pb(OAc)_2$. The results are presented in Table 5.1. In agreement to studies previously reported [61,62,85,88], all phosphors emitted the highest RTP intensities in the presence of iodide. All compounds exhibited very similar phosphorescence excitation and emission wavelengths for each heavy atom tested. Because of the broad nature of RTP bands, the individual SSRTP detection of these phosphors in a mixture requires some kind of previous separation.

Flow Rate Effect. The phosphorescence intensity of caffeine, theophylline, and theobromine presented significant variations with the

Table 5-1 Room Temperature Phosphorescence Characteristics of Caffeine Theophylline and Theobromine on Filter Paper External Heavy Atom Effect

Analyte[a]	Heavy Atom	λ_{ex} nm	λ_{em} nm	I_T[b,c]	I_B[b,d]	I_{net}[e]
Caffeine	I^-	275	451	204	63	141
	Tl^+	272	508	305	264	41
	Pb^{2+}	255	503	298	250	48
Theophylline	I	273	448	149	50	99
	Tl^+	262	510	251	213	38
	Pb^{2+}	296	508	272	232	40
Theobromine	I	278	451	230	44	186
	Tl^+	265	511	210	200	10
	Pb^{2+}	287	505	232	216	16

[a] Concentration used 10 $\mu g \cdot ml^{-1}$

[b] Average of three triplicates

[c] I_T means phosphorescence intensity of the analyte plus heavy atom (arbitrary units)

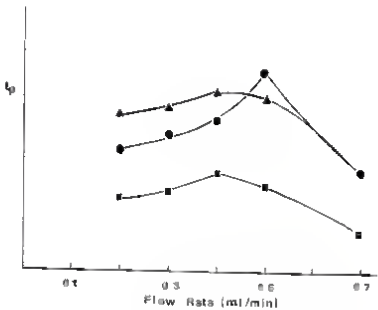
[d] I_B means phosphorescence intensity of the heavy atom blank (arbitrary units)

[e] I_{net} means net phosphorescence intensity = $I_T - I_B$

flow rate of the mobile phase The possible reasons for this phenomenon were discussed in Chapter 4 when similar variations were observed in the HPLC-SSRTP analysis of several polynuclear aromatic compounds Figure 5-1 shows the SSRTP response of caffeine theophylline and theobromine versus the flow rates tested The highest RTP signals were obtained between 0 4 and 0 5 mL/min Considering the reduction in the RTP signal response of theobromine (approximately 30%) when the flow rate was varied from 0 5 to 0 4 mL/min and the advantage of shorter analysis time we chose 0 5 mL/min as the flow rate to perform the separation with this flow rate s separation time of 8 minutes per sample was achieved The total analysis time however depended on the speed of the moving paper strip As mentioned in Chapter 4 the transport of the eluted compounds from the exit of the nebulizer to the sample compartment of the spectrophosphorimeter induced a delay time By increasing the filter paper speed, this delay time can be reduced resulting in a shorter analysis time

Filter Paper Speed Effect In order to obtain the shortest possible analysis time with the automated SSRTP detector we started the RTP measurements at the fastest filter paper speed supported by the AutoAnalyzer continuous filter (20 cm/min) Unfortunately at this substrate speed we were not able to detect RTP emission from any of the studied compounds For a constant spraying flow rate the amount of analyte deposited per cm^2 of filter paper only depends on the substrate speed At the fastest filter paper speed the analyte deposition is performed over the longest section of substrate resulting in the smallest amount of analyte deposited per cm^2 of filter paper In addition at 20 cm/min filter paper speed the drying time is the shortest and is responsible only spends approximately 2 min in the drying chamber Attributing the lack of RTP signals to the insufficient amount of analyte sprayed per cm^2 of filter paper and possible moisture quenching due to short drying times we reduced the speed of the filter paper strip Several substrate speeds ranging from 5 5 to 16 cm/min were tested for highest RTP intensities Figure 5 2 shows the SSRTP chromatograms obtained at three different

Figure 5-1 Solid substrate room temperature phosphorimetry peak area (I_p) variations of theobromine •, theophylline ▪ and caffeine ▲ with mobile phase flow rate variations. Mobile phase methanol/water 35/65 v/v. Sample size 600 pg of each compound injected volume 20 µL.


I_p
0.1
0.3
0.6
0.7
Flow Rate (ml/min)

paper speeds of a synthetic mixture containing theophylline theobromine and caffeine The mobile phase employed for separation was a 35 65 v/v methanol water solution At a filter paper speed of 16 cm/min (Figure 5-2a), a partially moisturized sample was still presented to the SSRTF detector In addition not enough analyte per cm^2 of substrate was probably sprayed to produce acceptable RTF signal intensities Higher RTF signal intensities were observed at 5 5 cm/min filter paper speed (Figure 5-2b), when the analytes spent approximately 4 min and 40 s in the drying chamber However, at such a low filter paper speed, the analyte diffusion on the substrate increased due to the high volume of mobile phase sprayed in a short section of filter paper strip As a consequence broadened and asymmetric peaks were observed resulting in a decrease in the chromatographic efficiency As a compromise between sensitivity and peak profiling a paper speed of 11 1 cm/min gave the best results for SSRTF detection (Figure 5-2c) By employing this substrate speed a delay time of approximately 7 min and 17 s was observed in the SSRTF chromatograms The total HPLC-SSRTF analysis time was about 15 min and 17 s per sample All the SSRTF chromatograms presented in this chapter have been retrieved for the delay time (7 min and 17 s) It is important to point out however that the optimum substrate speed for maximum RTF sensitivity and chromatographic efficiency depends on the flow rate and the nature of the mobile phase The use of a more volatile mobile phase would require shorter drying times resulting in faster paper speeds In this respect, though the limiting factor of substrate speed would probably be the amount of analyte sprayed per cm^2 of filter paper This amount could be easily increased by working at higher flow rates which would result in the advantage of shorter analysis times

Chromatographic Band Broadening The peak efficiencies were obtained assuming Gaussian peaks and are listed in Table 5 2 As observed there was a considerable loss of efficiency (approximately 87%) when the SSRTF chromatograms were compared to those obtained by UV detection This efficiency loss is approximately 6 times highest than the one observed in the comparative analysis of several polynuclear aromatic compounds by HPLC

Figure 5 2 Solid substrate room temperature phosphorimetry response (I_p) of theobromine (1), theophylline (2) and caffeine (3) versus filter paper speed a = 16 cm/min, b = 5 5 cm/min c = 11 1 cm/min

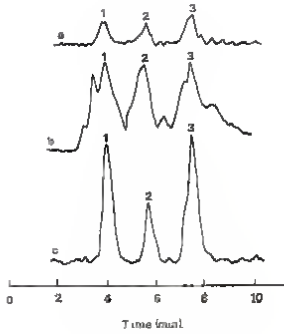

a
b
c
1
2
3
0
2
4
6
8
10
Time (min)

Table 5.2 Chromatographic Parameters Obtained by UV Absorption and SSRTF Detection in the HPLC Analysis of Theobromine, Theophylline and Caffeine

Solute	V_r[a] mL	k' [b]	Efficiency[c] UV plates	Efficiency[c] RTF plates
Theobromine	1.95	0.67	2156	187
Theophylline	2.65	1.27	2956	346
Caffeine	3.65	2.13	3960	573

[a]Retention volumes (V_r) obtained with a flow rate of 0.5 mL/min
[b]Capacity factors (k') calculated for a dial volume of 1.17 mL
[c]Efficiencies calculated using $N \sim 4\ (V_r/W_{0.60})^2$ in which $W_{0.60}$ is the peak width at 60% of the peak height expressed in volume units. Accuracy 20%

with SSRTF and UV detection (see Chapter 4). In that case, pure methanol was employed as a mobile phase. The chromatographic separation of caffeine, theophylline and theobromine required the use of a mobile phase composed by methanol:water 35:65 v/v. This eluent has a poorer volatility than methanol and requires longer drying times. As mentioned earlier, the presence of eluent on the substrate increases the diffusion of analytes and results in band broadening and loss of chromatographic efficiency. In future applications of the SSRTF detector for HPLC analysis, it would be convenient to choose the eluent based not only on its ability to separate the analytes of interest but also on its volatility.

Analytical Figures of Merit, AFOM. Table 5.3 compares the AFOM obtained with the UV absorption and the SSRTF system for HPLC detection. Both detectors were compared under experimental conditions for maximum SSRTF sensitivity and reproducibility. The limits of detection with the UV detector were calculated from the equation $c_L = 3/5\ (1_{p-p}/m)$ where 1_{p-p} is the peak-to-peak background noise and m is the slope of the calibration curve [49]. The SSRTF values were estimated by multiplying the equation above by the factor A_t/A_b, where A_t is the irradiated area in the sample compartment and A_b is the total area of substrate on which the analyte has been sprayed. The LOD of all studied compounds were in the nanogram level for both detectors. The SSRTF values were comparable with data previously reported in studies involving the optimization of parameters for SSRTF detection [87,88]. The same trend was observed for the UV LOD, which were in the same level of those obtained in the analysis of urine samples by HPLC-UV absorption detection [89-92]. The LDR achieved by SSRTF are approximately one order of magnitude larger than those obtained by UV absorption. This is in agreement with the LOD estimated, since the SSRTF values are from about 3 to 6 times lower than the UV ones. It is important to mention, though, that no efforts were made in order to obtain the upper limit of linear concentration of calibration curves. The relative standard deviations (based on triplicate measurements) of the UV absorption detection ranged from 3% (at medium concentrations) to 5% in low (1-10 ppm) and high (100-300 ppm) concentrations. As expected, the

Table 5 3 Analytical Figures of Merit Obtained for Theobromine Theophylline and Caffeine by High Performance Liquid Chromatography With Solid Substrate Room Temperature Phosphorimetry and UV Detection

Solute	LDR[a]	Slope[b]	Correlation Coefficient	LOD[c] (ng)
Theobromine	1 0x10^3 (1 0x10^2)	0 87(0 99)	0 996(0 999)	1 9(8 0)
Theophylline	1 7x10^3 (2 1x10^2)	0 88(0 96)	0 998(0 998)	2 4(7 5)
Caffeine	3 3x10^3 (2 0x10^2)	0 92(0 93)	0 995(0 997)	1 8(11 9)

All AFOM were obtained under experimental conditions for maximum SSRTP sensitivity and reproducibility The values listed in parenthesis correspond to the AFOM obtained with the UV detector
[a]Linear dynamic range (LDR) was estimated by dividing the upper linear concentration by the limits of detection
[b]Calculated from the curve log phosphorescence intensity (absorbance) versus log concentration
[c]Signal to noise ratio = 3 and volumes of 20 μL (SSRTP) and 8 μL (UV) were used to estimate the limits of detection (LOD)

SSRTF measurements presented higher RSD values than the UV ones and depended on the studied compounds. For theobromine, the RSD ranged from 8% (low concentrations) to 15% (high concentrations) with an improvement at medium concentrations (6%). Caffeine and theophylline showed RSD of 7.5 and 10% at low, medium and high concentrations, respectively.

SSRTF Detection of Caffeine, Theophylline, and Theobromine in Urine Samples

Fresh urine samples were spiked with known amounts of theobromine, theophylline and caffeine. After filtration, the samples were injected in the chromatographic system. Under the separation conditions employed, blank urine produces peaks in the UV chromatogram (see Figure 5-3). Although theophylline and caffeine are well resolved, theobromine appears between two urine peaks, seriously compromising the detectability of this compound. On the other hand, the SSRTF detector produces a clear chromatogram free from blank urine interferences. This is due to the essentially non phosphorescent nature of proteins at room temperature [93, 95]. Previous studies involving the SSRTF determination of PABA in urine samples without previous separation [94, 95] have shown that the nucleic acids responsible for the weak phosphorescence nature of urine do not cause significant contribution to the background signal of solid substrates. Since the magnitude of the SSRTF signals of the three drugs in spiked urine samples and aqueous solutions are within the standard deviations of measurements, and the blank response is similar in both cases, we believe that HPLC-SSRTF could be employed as an alternative method for the analysis of urine samples.

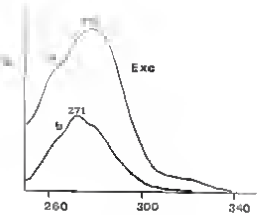
As mentioned in Chapter 4, the use of SSRTF detection offers the possibility of identifying the constituents of a stored sample at any required time. This is an attractive feature for areas where a permanent record of the analyzed sample is of interest. As a tentative means of evaluating this possibility for the specific case of caffeine, theophylline and theobromine, the filter paper strip corresponding to a chromatogram of a mixture of the three compounds was stored in a desiccator at room temperature covered with aluminum foil and in the

Figure 5 3 Spiked urine sample chromatogram obtained by UV absorption and solid substrate room temperature phosphorimetry detection (1) theobromine (2) theophylline (3) caffeine Mobile phase methanol/water 35 65 v/v Sample size 600 pg injected 20 µL

MOISTURE MEASUREMENT AS
Blank Pulse
Spiked Pulse
MOISTURE ASSAY
Spiked Urine
Urine
Time (min)

presence of anhydrous $CaSO_4$. The relative positions of the eluted phosphors on the filter paper strip were measured and marked with a pencil on the edges of the substrate. A new "chromatogram" of maximum RTP intensity versus distance was then obtained. One month later the filter paper strip was removed from the desiccator and placed on the filter paper rrack of the automated system for SSRTP identification and quantitation. The detection parameters were the same as those utilized in the measurements of the fresh sample. As expected the relative position of the eluted compounds on the substrate strip remained constant allowing their identification based on their phosphorescence signal versus distance. Searching for further identification, the filter paper strip was stopped or the maximum RTP signal intensity of each compound and the phosphorescence spectra were recorded. The excitation and emission spectra of these phosphors obtained with the fresh sample and one month later are shown in Figures 5-4, 5-5, and 5-6. Figure 5-7 shows the spectra of the blank obtained under the same experimental conditions. After one month of storage time significant RTP intensity reductions and changes in maximum wavelengths were observed in all analyte spectra. The background spectrum though remained approximately the same. Table 5-4 summarizes the modifications observed. There is a small shift to the blue (~ 5 nm) in the excitation spectra of all phosphors. The emission maxima were dislocated to a region of longer wavelengths and varied with the studied compounds. Possible variations in the intensity of the excitation source were investigated by comparing the RTP signal intensities of a phosphorescence standard (300 nm/615 nm) obtained at the moments of detection of fresh and one month old samples. No significant variations were observed, eliminating the necessity of signal normalization. However, RTP intensity reductions of about 2.5, 2.8, and 3.9 times were verified for theobromine, theophylline and caffeine respectively. Since a significant decrease in the background signal was not observed, there was a reduction in the signal to background ratio of all phosphors. These modifications obviously makes difficult the identification and quantitation of the studied compounds. It is important to notice though

Figure 5-4 Room temperature phosphorescence excitation (Exc) and emission (Em) spectra of theobromine in (a) fresh spiked urine samples sprayed on filter paper (b) after one month storage of filter paper substrate


a
271
Exc
b
271
260
300
340

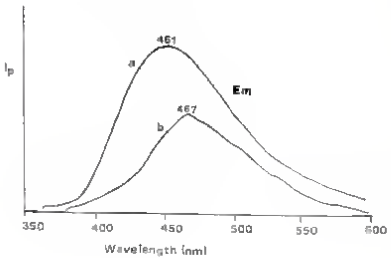

461
a
Em
b
457
350
400
450
500
550
600
Wavelength (nm)

Figure 5.5 Room temperature phosphorescence excitation (Exc) and emission (Em) spectra of theophylline in (a) fresh spiked urine samples sprayed on filter paper. (b) after one month storage of filter paper substrate

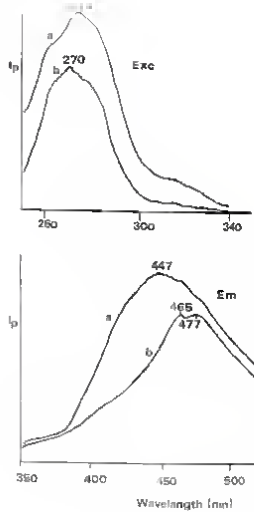

a
b
270
Exc
280
300
340
447
Em
465
477
a
b
350
400
450
500
550
600
Wavelength (nm)

Figure 5-6 Room temperature phosphorescence excitation (Exc) and emission (Em) spectra of caffeine in (a) fresh spiked wine samples sprayed on filter paper, (b) after one month storage of filter paper substrate

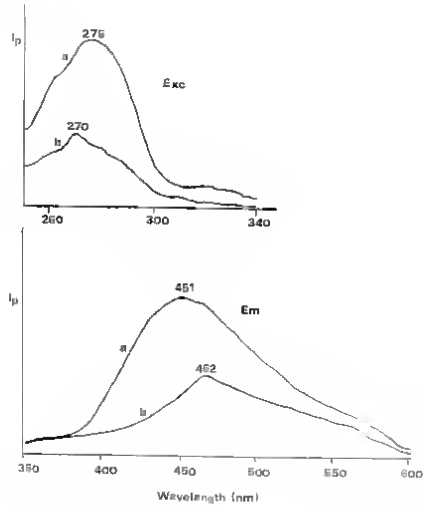

I_p
275
a
Exc
270
b
260
300
340
I_p
451
Em
a
462
b
350
400
450
500
550
600
Wavelength (nm)

Figure 5 7 Room temperature phosphorescence excitation (Exc) and emission (Em) spectra of urine samples sprayed on filter paper in the presence of 1M KI

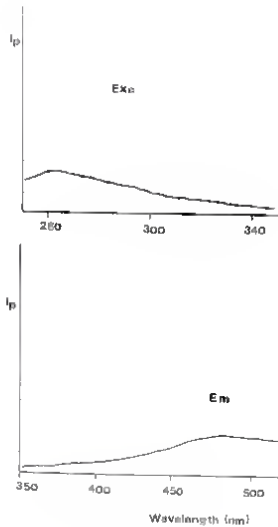

Ip
Exc
280
300
340
Ip
Em
350
400
450
500
550
Wavelength (nm)

Table 5 4 Effect of Storage Upon the Room Temperature Phosphorimetry Characteristics of Theobromine Theophylline and Caffeine

Compound[a]	Fresh Sample				One Month Later			
	λ_{exc} (nm)	λ_{em} (nm)	I_A[b]	S/B[c]	λ_{exc} (nm)	λ_{em} (nm)	I_A[b]	S/B
Theobromine	275	451	217	7 0	271	467	88	3 0
Theophylline	274	447	56	2 6	270	465[d] 477	20	1 5
Caffeine	275	451	172	5 8	270	462	44	2 ?
Blank								

[a]Concentration 10 μg/mL[-1]

[b]Phosphorescence intensity (arbitrary units) obtained by $I_A = I_{A+B} - I_B$ where I_{A+B} is the intensity signal of the analyte + blank and I_B is the blank signal I_{A+B} and I_B were measured at wavelength of maximum [illegible] intensity presented by each analyte in the fresh sample

[c]Signal to background ratio (S/B) calculated dividing I_{A+B} by I_B

[d]λ_{em} = 465 nm was employed to detect I_{A+B} and I_B

that no attempts were made to optimize the storage conditions. The presence of moisture in the desiccator could be to some extent responsible for the reductions observed in the RTF signals. Storage of the filter paper strips under a dry atmosphere of an inert gas could minimize moisture effects. The shifts observed in the maximum emission wavelengths suggest that sample degradation occurred on the filter paper strip. This possibility stresses, once more time, the necessity of a careful study involving the search for optimum storage conditions. Keeping the filter paper strip at low temperature could minimize degradations of the samples.

Conclusions

In this chapter, we have demonstrated the possibility of using HPLC-SSRTF as an alternative method for the quantitative analysis of caffeine, theobromine and theophylline in urine samples. APOM comparable to those obtained by UV absorption detection were achieved by SSRTF analysis. The experimental conditions employed produced UV chromatograms with urine peaks that seriously compromise the determination of theobromine. The non phosphorescent nature of urine, however, allows the SSRTF detection of theobromine free from blank interferences.

The feasibility of using SSRTF chromatograms as permanent records of analyzed samples strongly depends on the search of appropriate storage conditions. In the specific case of caffeine, theophylline and theobromine, the storage of filter paper strips in a desiccator at room temperature was not effective enough to avoid degradation of the compounds. This fact however, does not exclude the possibility of employing HPLC-SSRTF as a confirmation method for caffeine abuse by athletes. The LOD obtained by SSRTF allows the detection of lower concentrations of caffeine than the upper limit established by the MC (12 μg/ml). Since a small number of urine samples per sports event usually requires the verifications of positive screening tests, the longer time necessary for SSRTF analysis does not represent a serious disadvantage when the method is compared to UV absorption detection.

CHAPTER 6

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

During the last two decades, SSRTP has been considered among the phosphorimetry techniques as the most suitable approach to be applied in routine analysis of a large number of samples. The simplicity of its sampling procedure, eliminating the need for cryogenic equipment and for deoxygenation of solvents, and the possibility of automation have been pointed out as the most attractive features over LTP, MS-RTP, and RTPL in applications where the samples are small and precious, such as clinical chemistry, biochemistry and medicine, the small sample requirement of SSRTP associated to its high sensitivity and selectivity are equally important.

Although it is the simplest phosphorimetry technique to work with, its application to routine analysis of a large number of samples has been limited by the manual procedure commonly employed in SSRTP analysis. Sample preparation involves several manual steps which can lead to appreciable random errors, require some degree of experimenter's expertise, and consumes between 6 to 10 minutes per sample. The new automatic system developed in these studies has eliminated the manual steps and allows performance of 16 and 20 measurements per minute. By using appropriate nebulizers, small volumes of samples are conveniently sprayed on filter paper substrate (~5 $\mu L/cm^2$) permitting its utilization in areas where the availability of samples is limited. The possibility of using heavy atom solutions at their optimum concentration range, and the achievement of APOM comparable to those obtained by manual SSRTP procedures, suggest that the proposed system is a convenient approach to develop the full potential of the technique as an analytical tool for routine analysis.

The versatility of the two nebulizer automatic system allows to perform SSRTP analysis in a continuous mode. This is an attractive feature for its application in flowing systems. By employing FIA as a continuous sample introduction technique calibration curves were easily obtained with only one or two stock analyte solutions. When associated with HPLC, its performance was comparable to UV absorption detection. The high selectivity of SSRTP makes the proposed system a useful tool for liquid chromatographic separations. By choosing the appropriate set of measurement wavelengths, individual components of mixtures partially resolved by HPLC can be selectively determined. Identification of compounds either from the retention times or their RTP spectral characteristics is possible, showing the potential of the SSRTP detector for qualitative purposes. If needed, its selectivity can be improved by SEHAP. By using the appropriate combination of heavy atom solutions and excitation wavelengths it is possible to enhance the phosphorescent signal of a target compound in the presence of other phosphors. Time resolved phosphorimetry can also be used to improve the selectivity of the new system. Its application, however, is restricted by the similarity of phosphorescence lifetimes at room temperature. Synchronous excitation phosphorimetry should also be considered as a possibility of improving the selectivity of the SSRTP system, but its employment requires the use of a detection unit different than the one utilized in these studies.

When compared to HPLC-MSRTP, HPLC-SSRTP offers several advantages. It can be applied to a wider variety of phosphors, since more compounds have shown phosphorescence on solid substrates than in micelle stabilized solutions. The emission of phosphorescence on solid substrates does not require deoxygenation of samples, which leads to a simpler procedure and shorter analysis time. Finally, the number of publications involving SSRTP studies exceeds MSRTP published data. This fact facilitates the optimization of parameters for a larger number of compounds when SSRTP is applied as a HPLC detection techniques.

The use of the SSRTP detector for HPLC analysis is feasible in all areas where phosphorescent compounds need to be determined. Its utilization however is particularly interesting in those areas where a permanent record of the analyzed samples is of interest. The storage of samples sprayed on filter paper strips offers the possibility of identifying the constituents of the samples at any required time. The relative position of the eluted compounds on the substrate strip allows the identification based on phosphorescence signal versus distance. Further identification based on RTP spectral characteristics though strongly depends on the search of appropriate storage conditions. Future work needs to be done in this area seeking optimum storage conditions for compounds of interest.

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BIOGRAPHICAL SKETCH

Andres Dobal Campiglia, son of Mario Dobal Martinez and Delia Nally M. Campiglia de Dobal, was born February 16, 1960, in Las Piedras, Uruguay. In 1974, he moved with his family to Brasilia, Brazil, where he completed his high school studies. He entered University of Brasilia, Brasilia, DF, in March 1978 and received the degree of Bacheler of Chemistry in November 1982. In March 1983, he entered graduate school in chemistry at University of Brasilia. In 1984, he was hired by the Chemistry Department of that university as an assistant professor. The Degree of Master of Chemistry was received from the University of Brasilia in June, 1985, where he continued as an assistant professor until July 1986. After being awarded with a four year fellowship from Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior - CAPES to continue his studies in chemistry, he entered the graduate program in chemistry at the University of Florida in August, 1986, and received the degree of Doctor of Philosophy in June, 1990. Andres D. Campiglia is currently an associate professor at the Chemistry Department of the University of Brasilia.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

James D. Winefordner, Chairman
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